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DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF ARBACIA PUNCTULATA. II. EFFECT OF THE MEDIUM ON RESPIRATION

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Introduction

The first section (Hayashi, 1945) of this investigation presented evidence for the existence of a factor in the seminal fluid of Arbacia which influences the duration of fertilizing capacity of the sperm of the same species. The experimental results indicated that the factor was adsorbed on the sperm surface, and subsequently was lost into the surrounding medium. Since Gray (1928a) has shown a relation between "mechanical crowding" of sperm and the length of metabolic life of sperm, the question at once arose as to whether the seminal fluid factor influenced the fertilizing power of sperm through an effect on the metabolism of sperm. Consequently, a series of experiments was done investigating the effects of seminal fluid on the respiratory activity of sperm.

Materials and Methods

The microrespirometer used for these experiments was a compensating type, a modification of Krogh's respirometer.[1] It consisted of two similar vessels of conventional design connected through a common U-shaped manometer. To remove the carbon dioxide, filter paper wet with 20 per cent KOH was placed in the manometer vessels out of contact with the respiring cells. The manometer vessels were immersed in a constant-temperature bath maintained steadily at 25° C. The shaker, upon which the microrespirometers were mounted, moved to and fro in a straight line a distance of 15 centimeters. A steady rate of 40 cycles per minute thoroughly agitated the respiring sperm suspensions. The sensitivity of the respirometer was such that a respiratory rate of one mm.^{3} of oxygen consumed per hour could be detected in ten-minute readings.

Two points of difficulty were encountered in the course of these experiments. First, capillary action prevented the easy transfer of any liquids from the side-arm into the respirometer vessel. Second, the effect of dilution on the respiration of sperm was found to be very rapid, and therefore difficult to measure. To overcome these difficulties, a measured amount of packed sperm was placed on the glass wall

[1] Kindly lent by Dr. Titus C. Evans, formerly of Iowa State University.

of the manometer vessel above the point reached by a given amount of liquid in the vessel, when this liquid was agitated by the movements of the shaker. The packed sperm remained in place by their own adhesive action. When the time came to dilute the sperm in the medium to be studied, the adhered sperm on the side of the vessel were washed down into the liquid by a slightly greater agitation of the manometer vessel.

This technique overcame the first of the above-mentioned difficulties satisfactorily, but only partly the second. To wash down and to disperse completely the sperm cells in the medium required time in the order of minutes. Since, during this period, changing numbers of cells were being affected by the medium, neither the total respiration nor the total respiratory rate could be accurately measured. This effect was minimized by manipulation of the vessels and by extrapolation of the respiration curve.

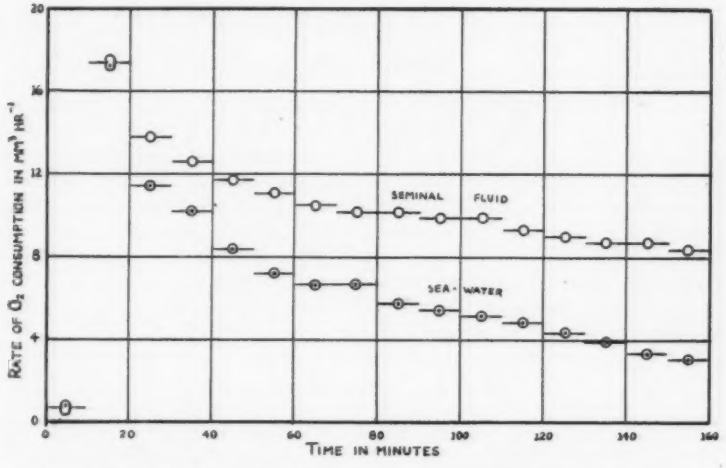

FIGURE 1. Effect of seminal fluid, sea water on respiratory rate of sperm.

The pH of the medium in the experiments here presented was not checked after each run, although it was known before the run, and has been published elsewhere (Hayashi, 1945). In later, similar experiments (to be published) the pH was adjusted to equality and checked before and after each run. In the time of the run, no change in pH occurred, and the results did not differ from those presented.

It was noted that the sperm in all the respiration experiments, irrespective of the medium used, and in the concentrations of these experiments, were dead at the end of five hours. A sharp rise in the rate of oxygen consumption occurs at about this time, due, probably, to disintegration of the sperm cells caused by the constant shaking of the manometers. This same effect was noted by Gray (1928b, p. 350). This effect afforded no trouble in the interpretation of the results, however, for it occurred uniformly in all sperm suspensions, and the pertinent data were obtained before the end of the five-hour period.

Experiments and Results

For the first experiments, the respiration of sperm in seminal fluid was compared with the respiration of an equal amount of sperm in sea water. Each suspension contained 0.00155 cc. of packed sperm per cc. of medium. The contrasting changes in the respiratory rates of the two suspensions are shown in Figure 1. Using the same data, the total oxygen consumed was plotted as a function of time, to produce the curves of Figure 2.

The experiment showed that the seminal fluid did not affect the sharp increase in respiratory activity immediately following the dilution of the sperm. The main effect of the seminal fluid was to regulate, or delay, the sharp drop in respiratory rate shown by the sperm in sea water. Figure 1 also showed that the total amount of energy expended by equal amounts of sperm in the same length of time was greater when the sperm were suspended in seminal fluid.

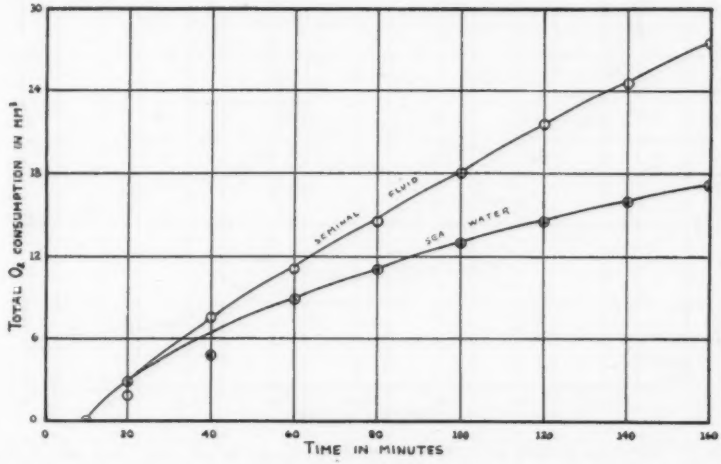

Figure 2. Total oxygen consumption as a function of time for sperm in seminal fluid and sea water.

As a further check on the effect of dilution on the respiration of sperm, one of Gray's (1928a) experiments was repeated, with both seminal fluid and sea water. A measured quantity of packed sperm (0.00155 cc.) was placed on the wall of the respiration chamber, and 1.0 cc. of medium was placed on the bottom of the vessel. In addition 0.5 cc. of medium was placed in the side arm, and this amount of medium was "dumped" over into the respiration chamber at an appropriate time. The results of the experiment are shown in Figure 3.

The results confirmed Gray, but compared with his results, the degree of rise in the respiratory rate at the second dilution was less. When the sperm concentrations used by Gray were compared with those used here, it was seen that the present sperm suspensions were far more dilute. Therefore, the results were taken to mean that the concentration of sperm in this experiment was near the

upper limit to obtain the maximum burst of energy at first dilution from the amount of sperm used. In other words, greater original dilution of the packed sperm would not result in very much more initial activity.

This interpretation was partly confirmed by the following experiment. Lillie (1913) had found that egg water stimulated sperm to greater activity, and Gray (1928c) concluded from his experiments that egg water stimulated sperm to greater respiratory activity. However, Gray had used egg water as the first and only diluent for the sperm. In the following experiment, by contrast, the egg water was used as a diluent after the initial burst of activity induced by the original dilution of the medium.

The same procedure as the preceding experiment was employed. Instead of the respective media in the side arms, however, egg water was used, and this was

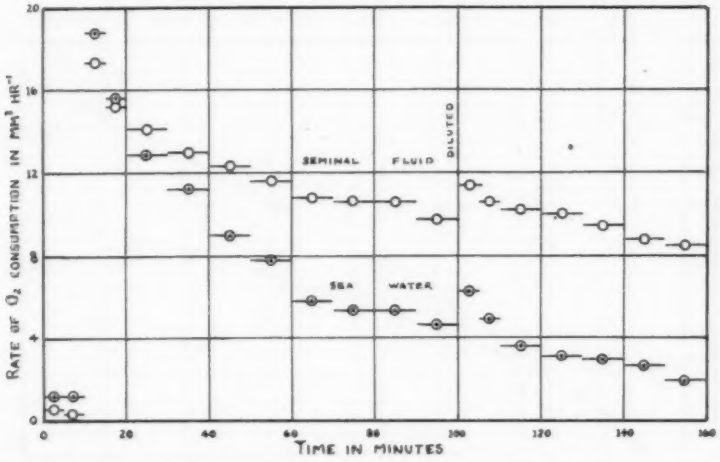

FIGURE 3. Effect of dilution by respective media on sperm in sea water and seminal fluid.

"dumped" in at the appropriate time. The results are given in Figure 4. Study of Figure 4 showed that, after the original burst of activity, egg water, far from stimulating the sperm, seemed to inhibit them. The effect was probably due to the agglutination of the sperm, and seemed to overcome the slight stimulation due to dilution shown in Figure 3.

DISCUSSION

Analysis of the effect of the seminal fluid on sperm respiration

The respiratory rate of sperm in seminal fluid is compared with that of sperm in sea water in Figure 1. The seminal fluid does not seem to influence the initial steep rise in activity due to dilution. There was some variation shown in this first burst of respiration, but a check of all the runs made showed that the variations were not significant. In some cases the sperm in seminal fluid showed more intense

activity at dilution; in others, the sperm in sea water were more active at dilution. The variations were caused probably by differences in the rate at which the sperm were washed from the sides of the respiratory vessels.

The effect of the seminal fluid is on the "senescent period" following the initial respiratory rise. The seminal fluid seems to prevent the rapid decline of the metabolic rate evident in the sea-water control. The effect of the seminal fluid on the respiration of sperm thus parallels the effect of seminal fluid on the fertilizing power of sperm. In both cases, the seminal fluid maintains the sperm at a high functional level for a longer time than does sea water. A comparison of the areas under the curves shows that sperm in seminal fluid expend more energy than an equal amount of sperm in sea water during the course of their active lives. There are two possible explanations for these results. It is possible that the sperm in seminal fluid utilized

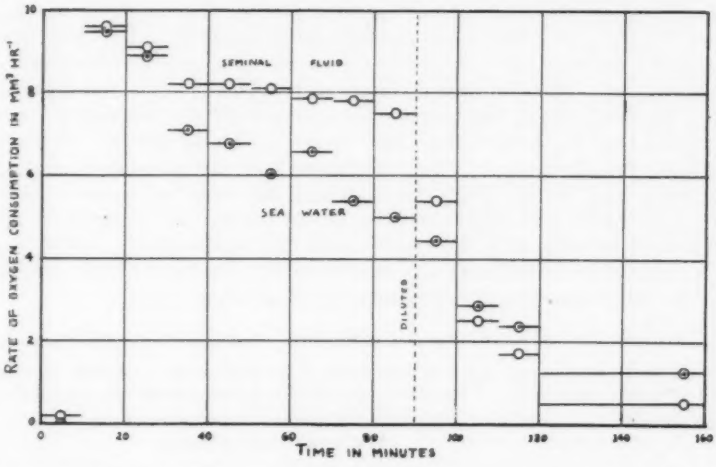

FIGURE 4. Effect of dilution by egg water on sperm in sea water and seminal fluid.

their internal store of fuel more completely than the sperm in sea water. The second possibility is that the seminal fluid is serving as a source of fuel for the sperm. A clue to these possibilities was sought by utilization of some of Gray's analytical methods.

Gray (1928b) originally advanced the view that the sperm cell was a tiny combustion engine with a limited supply of fuel. From his original assumption, Gray derived the equation

$$\text{Activity} = \frac{dx}{dt} = k(a - x) \qquad \text{(Equation 1)}$$

which, integrated, gave

$$k = \frac{1}{t} ln_e \frac{a}{a - x}. \qquad \text{(Equation 1a)}$$

In this equation, x represented the amount of fuel used up in time, t; a, the initial, total amount of fuel in the sperm cell; and k, the activity produced by each unit of fuel consumed. Gray found that this "law of exponential decay of a homogeneous population" did not fit the experimental results. He, therefore, expanded the equation with a consideration of a heterogeneous population, and derived, as one form of the equation,

$$x = O_2\left(\frac{t}{\alpha + t}\right), \qquad \text{(Equation 2)}$$

in which O_2 represented the total oxygen consumed during active life; α, another constant; and x and t, possessing the same meanings as before. Gray found that this equation could be made to fit the experimental results, and concluded that the sperm population was heterogeneous in a special way, and that its activity decreased because of the depletion of an internal supply of energy. The notion of heterogeneity was used as a basis for a later, more detailed mathematical analysis (Gray, 1930).

The above methods were applied to the results of a number of experiments of this investigation. At two different values of time t in a single run, experimental values of the total oxygen consumption were arbitrarily selected, and the values of the constants k, a, O_2, and α in equations 1a and 2 were calculated. The values of the constants thus derived were then substituted back in the equations and the theoretical values of x for the entire run calculated and compared to the experimental values. The results showed that, with the proper selection of points, both equations 1a and 2 could be made to fit the experimental data rather closely, but other points could be selected to demonstrate that neither of the equations fit the experimental data. The latter case, in a typical experiment, is shown in Table I.

TABLE I

Comparison of the experimental and theoretical values of the total oxygen consumption of sperm in sea water and seminal fluid. Theoretical values calculated from equation 1a and equation 2

Time (minutes)	Total oxygen consumed in mm.³					
	Sea water			Seminal fluid		
	Exp.	$k = .01275$ $a = 12.24$	$O_2 = 15.25$ $\alpha = 71.15$	Exp.	$k = .00876$ $a = 16.31$	$O_2 = 22.54$ $\alpha = 135.7$
0	0.00	0.00	0.00	0.00	0.00	0.00
10	1.05	1.45	1.87	0.95	1.36	1.55
20	2.07	2.75	3.32	1.99	2.62	2.90
30	3.09	3.89	4.50	3.08	3.77	4.08
40	4.06	4.89	5.46	4.06	4.83	5.13
50	4.96	5.77	6.26	5.06	5.78	6.07
60	5.84	6.55	6.94	6.04	6.66	6.91
70	6.68	7.23	7.53	6.97	7.47	7.67
90	8.08	8.36	8.49	8.60	8.90	8.99
100	8.70	8.86	8.88	9.37	9.52	9.56
110	9.23	(9.23)	(9.23)	10.09	(10.09)	(10.09)
210	11.41	11.40	11.37	13.80	13.72	13.69
220	11.50	(11.50)	(11.50)	13.94	(13.94)	(13.94)
255	11.76	11.76	11.90	14.31	14.56	14.71

It may be noted that the points selected are those covering the end of the run, and the data of this portion fit the experimental values rather closely, whereas wide variation is evident in the early portions of the run. Points selected near the beginning of the run, in contrast, would show a fit in that portion of the run, and variation at the end. Intermediate points, of course, would show an intermediate fit.

From such analyses, these conclusions may be drawn. (1) There is no need to assume heterogeneity of the sperm population whether in sea water or seminal fluid. (2) The simple "law of exponential decay" is not adequate to explain sperm activity; the decrease in activity of the sperm cells with time is not due to the exhaustion of an internal supply of fuel alone, whether the sperm cells are in sea water or seminal fluid.

Since sperm cells in sea water show a decline in activity whose controlling factor is not the exhaustion of their internal source of fuel, it is possible that even at the end of active life in this medium, the sperm cells contain unused, potential energy. The utilization of this unused energy by the sperm in seminal fluid would explain the increased oxygen consumption of the sperm cells in seminal fluid. There is, however, the possibility that the sperm in sea water reach the end of active life because of starving *plus* other factors. The question of nutrition by seminal fluid, therefore, is unsettled by the above analyses, although strong possibilities are afforded.

Gray (1928b) analyzed the senescence of spermatozoa from still other considerations. He assumed that the cause of the death of the spermatozoon was the accumulation of products of metabolic activity inside the sperm cell. This would render inactive part of the active system originally present in the cell.

$$\text{Active system} + \text{product of activity} \rightleftarrows \text{inactive system.}$$

Based on this concept Gray derived an equation which he called the "theory of autointoxication."

$$x = \sqrt{\frac{2Ka}{b}}\sqrt{t}. \qquad \text{(Equation 3)}$$

Here x, a, and t designated the same quantities as before, and K and b were constants. Equation 3, based primarily on the assumption of a homogeneous population, showed that the amount of fuel, x, consumed in time t was directly proportional to the square root of t. Therefore, the total oxygen consumed should show a straight-line relationship to the square root of the age of the active suspension. Gray found that this relationship did not hold for sperm in sea water, for toward the end of active life the points fell below the expected values. He concluded that part of the active system was not only being inactivated, but also being irreversibly destroyed.

Equation 3 was applied to the experimental figures of the present investigation. When the total oxygen consumption of the sperm in sea water is plotted against the square root of the time, the results confirm Gray's findings, for, toward the end of the run, the points fall below the expected values. (The variation from the straight-line relationship at the beginning of the run is due to the time requird for the cells to be completely dispersed in the medium.) For the sperm in sea water, therefore, an irreversible destruction of part of the active metabolic system is taking place to-

ward the end of the run (Fig. 5). In contrast, the straight-line relationship is followed for a much longer time in seminal fluid in an equivalent run (Fig. 6). In some of the experiments, a departure from the straight-line relationship occurred in the seminal fluid, but in all cases, this departure occurred very much later from the beginning of active life. In the seminal fluid, therefore, the irreversible destruction of the active system is much delayed, as compared to the case of the sperm in sea water. The decay of activity of the sperm in seminal fluid is apparently due, at least for a great part, to a process of autointoxication.

The difference between the metabolism of sperm in sea water and in seminal fluid apparently lies in the fact that some sort of metabolic system is kept intact for a longer time when the sperm cells are suspended in seminal fluid. The sperm and a factor in the seminal fluid, therefore, constitute a closed system. In sea water,

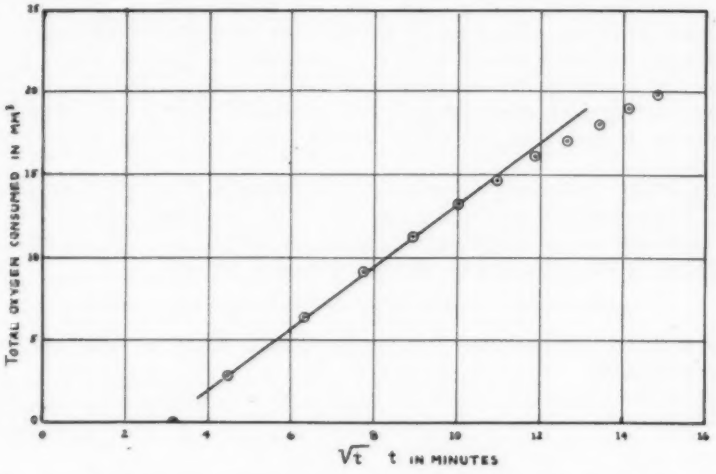

FIGURE 5. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in sea water.

part of this closed system is destroyed quite early during the active life of the sperm.

The above findings, supported by the knowledge that seminal fluid contains no reducing sugars (Hayashi, 1945), lends further support to the idea that the effect of seminal fluid on sperm respiration is not due to the replenishment of a store of energy, but rather to the maintenance of a metabolic system. Other experiments (unpublished data) measuring the R.Q. of sperm cells both in sea water and seminal fluid show a characteristic carbohydrate metabolism for these cells, further supporting the contention that the seminal fluid does not provide added nutrients for the sperm cells. It may be concluded that, although there is the possibility that sperm can be nourished experimentally, it is most important for future experimentation that the system, of which the sperm cells are only a part, be kept intact. Workers who have diluted sperm cells in sea water have diluted not only cells, but, also, system.

Both the fertilizing power (Hayashi, 1945) and the respiratory activity of sperm are maintained by a common factor, the seminal fluid. As a first possibility, the role of the seminal fluid factor may be considered fundamental to the respiratory activity only, the fall in fertilizing power being a manifestation of the loss of activity of the sperm cells. In other words, the fall in fertilizing power is only a secondary effect in relation to the seminal fluid factor. The second possibility is that the seminal fluid plays a part directly in both fertilization and respiration. Lack of data does not permit the choice of these possibilities. However, the fact that a seminal fluid factor influences sperm respiration is implicit in both possibilities.

A mechanism, tentatively proposed earlier as the wearing off of a protein substance from the surface of the sperm cell, was adequate to explain the loss of fertilizing power of sperm in sea water. The same mechanism is also applicable to the

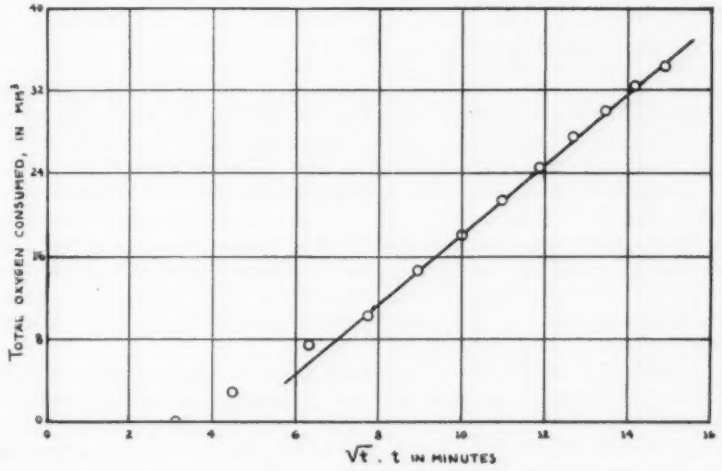

FIGURE 6. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in seminal fluid.

results of respiration studies, if the assumption be made, that the removal of each molecule of protein substance from the surface of the sperm cell releases to the sperm cell a finite amount of the total internal energy. The rate of sperm respiration thus would be controlled by the rate of removal of substance from the sperm surface, which is in turn controlled by other factors. The principal factor affecting the rate of removal of a sperm-surface-substance is the "mechanical crowding" factor studied by Gray (1928a), who found that sperm cells exhibited a burst of activity when diluted. This was confirmed in the present study (Figs. 1, 3, 4). According to the mechanism proposed, the rate of loss of sperm-surface-substance is inhibited when the cells are in a crowded condition, but when relatively far apart, the removal of surface-substance is enhanced.

The removal of sperm substance from the cell surface presumably constitutes the irreversible destruction of part of the active system noted in Fig. 5. In seminal fluid,

however, the replacement of the surface-substance would delay the onset of this irreversible destruction (Fig. 6), the senescence of the sperm cells being conditioned by internal autointoxication, and perhaps also by depletion of the internal energy. It would seem that for the sperm cells in sea water, the destruction of part of the system, in addition to autointoxication and probable depletion of fuel, controls the fall in activity.

It is understood that the mechanism as outlined is tentative at best, but it serves as an explanation of the facts so far known. The facts fit well with the earlier results of the effect of seminal fluid on the fertilizing capacity of sperm. It may be concluded that possibly a common mechanism underlies the effect of seminal fluid on the fertilizing function and the respiration of sperm. The relationship between the surface-substance and the respiratory mechanism of the sperm cell was not investigated. The action of the surface-substance while attached to the sperm cell is possibly enzymatic.

Effect of egg water on sperm respiration

Egg water does not stimulate the respiration of sperm cells but seems to condition a sharp drop in the respiratory rate (Fig. 4). When egg water was added to sperm suspended in sea water and, also, to sperm suspended in seminal fluid, the effect was a sharp decrease in the respiratory rate. This decrease of metabolic activity upon dilution with egg water is in contrast to the effect of dilution with sea water and seminal fluid shown in Figure 3. The increase in respiratory rate brought about by dilution with sea water and seminal fluid shows that even at the dilution used, "mechanical crowding" was still apparently a factor inhibiting the respiration of spermatozoa. Dilution with egg water also relieved the "mechanical crowding," but the agglutinating effect of egg water apparently overcame the effect of dilution so that the respiratory activity decreased.

The stimulation of metabolism by egg water noted by Gray (1928c) and Carter (1930) is not confirmed in these experiments. However, it may be recalled that Carter (1931) had found no stimulation of ripe sperm by egg water. In addition, the egg water of the present investigation was added, not to undiluted sperm, but to sperm that had already been activated by an initial dilution. Therefore, it is possible either that egg water did not affect the respiration of sperm at all, or that the difference in the time of addition of egg water was the cause of the disparity of the results of Gray's experiments and the results of the present investigation.

Summary

1. Seminal fluid has the property of delaying the fall of respiratory activity of the sperm after the original burst of activity upon dilution.

2. Gray's "theory of exponential decay" is not adequate to explain sperm metabolic activity, whereas the "theory of autointoxication" fits the activity of sperm cells suspended in seminal fluid.

3. The seminal fluid delays appreciably the onset of an irreversible destruction of part of the metabolic system.

4. Fertilization studies have led to the formulation of a tentative mechanism based on the adsorption of a protein substance and its removal from the surface of the sperm cell.

5. The proposed mechanism also explains adequately the results of the respiration experiments. Therefore, it is concluded that a seminal fluid factor, by its action while on the surface of the sperm, influences both the fertilizing capacity and the respiratory rate of spermatozoa.

6. Egg water added to a sperm suspension after the original dilution, causes a sharp decrease in the respiratory rate.

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THE EFFECTS OF LITHIUM CHLORIDE ON THE FERTILIZED EGGS OF NEREIS LIMBATA

CATHERINE HENLEY

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Introduction

The action of the lithium ion on the eggs of certain animals has long been known. Using a number of lithium salts, Herbst (1892) obtained abnormal embryos of two types. In the first group, the endoderm was found not inside the body, but outside; these forms are designated "exogastrulae." Larvae of the second group showed an apparent conversion of ectoderm into endoderm so that in extreme cases, the whole blastula wall was endodermized (Herbst, 1892, 1893, 1943). Herbst concluded that this was a specific and typical affect of lithium, a view shared by Gurwitsch (1895), Morgan (1902), and other early investigators.

Studying the effects of 0.2 per cent to 1.0 per cent solutions of lithium chloride on frog and toad eggs, Gurwitsch found that in the resulting embryos, gastrulation was abnormal, but no exogastrulae were produced. Morgan (1902), using similar concentrations of lithium, obtained embryos in which the black hemisphere had sunk into the interior of the egg, but here again there was no definite indication of exogastrulation. Experiments by Holtfreter (1931) showed that amphibian exogastrulae could be obtained if the blastulae were treated with a modified Ringer solution. Further experiments to ascertain the effects of lithium on the amphibian egg were carried out by Bellamy (1919) who observed a number of cases of fusion of the lateral sense organs of the larvae, as well as abnormalities in gastrulation. Töndury (1938) treated urodele embryos in early stages of gastrulation with LiCl and described a high percentage of head and foregut abnormalities in the larvae. Still another effect of lithium on urodele eggs was observed by Cohen (1938) who treated early gastrulae or late blastulae and observed cases in which the myotomes formed a continuous sheet across the midline, separating the nerve cord and notochord. He also obtained embryos in which exogastrulation had occurred, seemingly as a result of the treatment with lithium.

The effects of LiCl on the eggs of the pond snail, *Limnaea stagnalis*, were studied by Raven (1942) who was able to produce forms which he designates as exogastrulae. He also describes a number of larvae in which varying abnormalities of the eyes were apparent. These experiments are of interest in connection with the present series because the egg of Limnaea, like that of Nereis, is an example of the so-called "mosaic" type of development.

A wide variety of chemical and physical agents has since been found to produce echinoderm exogastrulae, thus invalidating the theory of an ion specificity. These agents include hypotonic sea water, isotonic solutions of magnesium chloride and lithium chloride, and combinations of isotonic solutions of magnesium chloride, so-

dium chloride, potassium chloride, and calcium chloride as studied by Waterman (1932); solutions of nickel chloride and copper chloride (Waterman, 1937); lithium in low concentrations augmented by carbon monoxide (Runnström, 1935); and 97 per cent carbon monoxide with 3 per cent oxygen in the presence of light (Runnström, 1928a). MacArthur (1924) treated sea urchin eggs with calcium chloride, stale or diluted sea water, copper sulfate, mercuric chloride and potassium cyanide, and obtained exogastrulae. Using "auxin," glycogen and potassium chlorate, Motomura (1934) likewise obtained echinoderm exogastrulae.

Most of the eggs previously tested with lithium have been those of echinoderms and amphibians, both of which are characterized by an invaginative type of gastrulation. It seemed, therefore, that an egg showing a strictly epibolic form of gastrulation should be tested, in an effort to ascertain if the same effect could be produced. The following experiments were performed to study the effects of lithium on the fertilized eggs of the annelid, *Nereis limbata*.[1]

Methods

Gametes of the heteronereis form of *Nereis limbata* were obtained at Woods Hole from June to September, during appropriate phases of the moon. Usually the eggs from one female were sufficient for an average experimental series; they were inseminated according to the methods outlined by Just (1939) and were then washed with freshly drawn, filtered sea water.

The fertilized eggs were allowed to remain undisturbed for a period of 75 minutes after insemination; at the end of this time (shortly before the first cleavage), they were transferred with as little sea water as possible to a series of 25 cc. stender dishes, to which the various solutions of LiCl were then added. Appropriate controls were kept in all series, these being cultured in filtered aerated sea water which was changed at daily intervals. A stock solution of 0.54 M LiCl in distilled water was used for all experimental mixtures; such a solution is approximately isotonic in all dilutions with sea water. Experimental mixtures were designated according to the percentage of this stock solution used—e.g., a "15 per cent solution" indicates that 85 cc. of filtered aerated sea water were added to 15 cc. of the 0.54 M LiCl stock solution. About 15 cc. of the mixture were added to each stender dish; at intervals ranging from 15 minutes to 36 hours, the solutions were decanted carefully and the eggs washed in three or four changes of sea water. LiCl-sea water mixtures ranging from 2 per cent to 100 per cent were tested for varying periods of time. No attempt was made to control the room temperature, which varied from 20° C. to 28° C. However, all cultures were kept in moist chambers which stood in running sea water; the temperature of this sea water averaged about 20° C., and did not vary more than one or two degrees at any time during the entire series of experiments.

Less extensive test series were also conducted for purposes of comparison, using the eggs of Nereis from which the vitelline membrane had been removed, according to the method described by Costello (1945a).

[1] The author wishes to express sincere appreciation to Prof. D. P. Costello for advice and encouragement throughout the course of the investigation, to Prof. Viktor Hamburger and Dr. Charles Packard for use of laboratory space at the Marine Biological Laboratory during the summers of 1944 and 1945, to Prof. B. H. Willier for his kindly interest, and to Mr. John S. Spurbeck for valuable assistance with the drawings.

Results

The most striking characteristic of the experimental larvae is the complete absence of exogastrulation, or of any definite evidence of true vegetalization. Abnormal larvae occurred to some extent in almost all the cultures; these abnormalities included the absence of an apical tuft, lengthening of the prototrochal cilia (Fig. 2A), absence of these cilia in varying degrees (Fig. 2), absence or abnormality of the eye spots (Fig. 3), deficiencies in the number of prototrochal cells (Fig. 3), atypical seta sacs with derangements in the number and position of these organs in older larvae (Fig. 4), and abnormalities in the number and position of the oil droplets (Figs. 2C, 3D, 4B). Most of the experimental larvae had abnormalities of the anal and prototrochal pigments; these abnormalities included both the absence of pigment and an abnormal concentration of pigment granules in various regions of the larvae (Figs. 2, 3, 4, 5). The specific occurrence of these anomalies was roughly proportional to the severity of treatment, being far more marked in cultures which

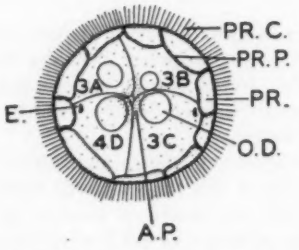

Figure 1. A normal Nereis trochophore at about 30 hours; polar view. Pr. C. = Prototrochal cilia; Pr. P. = Prototrochal pigment; Pr. = Prototrochal cell; O. D. = Oil droplet; A. P. = Anal pigment; E. = Eye spot; 3A, 3B, 3C, 4D = Entomeres. This and all subsequent text-figures are semi-diagrammatic, based on camera lucida drawings of the living larvae.

were exposed to higher concentrations for sublethal periods. No evidence of twinning was observed.

Complete data describing the results of treatment with the varying concentrations for varying periods are contained in Table I. It is evident that concentrations of 2 per cent to 4 per cent acting for periods up to 12 hours produce larvae which are almost completely normal; similar trochophores result when 5 per cent to 10 per cent solutions are applied for one to 5 hours. However, when allowed to act for periods of 5 to 24 hours, the same concentrations affect the eggs so that the resulting larvae show marked deficiencies in the number of prototrochal cells, and are usually completely devoid of the normal anal and prototrochal pigment. Abnormalities such as these occur quite commonly in all series; some extreme examples are shown in Figure 2. For purposes of comparison, a normal trochophore is shown in Figure 1. Accurate quantitative observations of the exact deficiency in the number of prototrochal cells were impossible in most cases.

Twelve per cent solutions acting for periods of 1½ to 5 hours result in trochophores which are essentially normal, but if allowed to remain on the eggs for 5 to 24 hours, bring about pigment and prototrochal anomalies of the type described above.

TABLE I

Effects of lithium treatment of Nereis eggs

Percentage[2]	Duration of treatment in hours	Appearance of 26–30 hour larvae
2%	1½	Normal
	5	Normal
	8	Mostly normal; some with abnormalities in number and position of oil droplets
	23	Mostly normal; some with abnormalities in number and position of oil droplets
3	8	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	10	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	24	Abnormal distribution of oil droplets, otherwise normal
4	1½	Normal
	5	Mostly normal
	8	Mostly normal
	12	Normal in form; somewhat sluggish in movements
5	14	Normal
	16	Mostly normal
	18	Mostly normal
	20	Some prototrochal and pigment abnormalities
	22	Pigment and prototrochal abnormalities
	26	Considerable variation in amount of pigment present
	28	Variations in amount of pigment present
	30	More marked pigment abnormalities than after 28-hour treatment
	32	Pigment and prototrochal abnormalities
	36	Dead
6	1½	Normal
	5	Normal, but sluggish in movements
	8	Mostly normal
	12	Normal, but sluggish in movements
	23	Quite abnormal; prototrochal and pigment abnormalities
7	1	Normal
	2	A very few cases with oil droplet deficiencies
	5	Some cases with oil droplet deficiencies
	24	Prototrochal cell deficiencies; sluggish in movements; pigment often absent
8	1½	Normal
	5	Some cases with prototrochal and pigment abnormalities
	8	More marked pigment abnormalities
	12	Severe prototrochal deficiencies; no pigment
	23	Severe prototrochal deficiencies; no pigment. Oil droplets abnormal in number and position. Many larvae without apical tuft

[2] Percentages in the table refer to percentages of 0.54 M stock solution of lithium chloride with sea water: 2 per cent = 2 cc. 0.54 M LiCl + 98 cc. sea water.

TABLE I—*Continued*

Percentage[a]	Duration of treatment in hours	Appearance of 26–30 hour larvae
10	1	Some localized deposits of pigment; some pigment present in most cases
	4	Some pigmented, some unpigmented. A few cases of abnormality in amount and position of anal pigment
	8	Pigment abnormalities; prototrochal cells seem fairly complete
	12	Pigment abnormalities; prototroch fairly normal
	14	No pigment; marked defects in number of prototrochal cells
	16	No pigment; marked defects in number of prototrochal cells
	18	No pigment, no cilia, no apical tuft
	20	No pigment, no apical tuft. Abnormalities in number and position of oil droplets
	24	No pigment, no cilia, no apical tuft. Abnormalities in number and position of oil droplets
	26	No pigment; marked prototrochal deficiencies. No apical tuft; oil droplets very abnormal in number and position
	28	No pigment; prototrochal defects marked. Oil droplets abnormal in number and position
	36	Dead
12	1½	Fairly normal; some abnormalities in number of prototrochal cells and oil droplets
	5	Many deficiencies of pigment and prototroch; oil droplets abnormal in number and position
	8	Pigment deficiencies; prototroch and oil droplets abnormal in number and position
	12	Some abnormalities in distribution of oil droplets; no pigment. Prototrochal deficiencies; no apical tuft
	23	Dead
14	1½	Mostly normal; some pigment deficiencies
	5	No pigment; marked prototrochal deficiencies
	8	No pigment; prototrochal deficiencies; no apical tuft
	23	Dead
15	1	Mostly unpigmented; prototrochal deficiencies. Anal pigment present in many cases. Oil droplets abnormal in number and position
	2	Unpigmented; prototrochal deficiencies; anal pigment present in some cases. Oil droplet abnormalities
	4	More marked pigment defects; severe prototrochal deficiencies. Some few cases of endodermal extrusion
	6	Highest incidence of endodermal extrusion: 2%–7%
	8	Somewhat fewer cases of extrusion than in 6-hour culture
	10	Some few cases of endoderm extrusion
	12	A very few cases of endodermal extrusion
	14	No pigment; marked prototrochal defects. No apical tuft
	16	No pigment; prototrochal defects; no apical tuft
	20	No cilia, no pigment, no apical tuft. Severe prototrochal deficiencies
	24	No pigment, no cilia, no apical tuft

TABLE I—*Continued*

Percentage[2]	Duration of treatment in hours	Appearance of 26–30 hour larvae
17	1	Anal pigment present; prototrochal pigment absent in many cases. Considerable number of oil droplet abnormalities
	2	No pigment; oil droplet abnormalities; prototrochal deficiencies; no apical tuft
	3	No pigment; oil droplet abnormalities in number and position. Prototrochal deficiencies, no apical tuft
	6	Marked oil droplet deficiencies and abnormalities in position; no pigment. No apical tuft; prototrochal cell deficiencies
	8	Many amorphous forms; no cilia; abnormalities in number and position of oil droplets
	10	No cilia, no pigment, no apical tuft
	14	Dead
20	1	Normal
	2	Pigment abnormalities; some localization of pigment
	4	No pigment; prototrochal cell deficiencies; apical tuft apparently missing; oil droplets abnormal in number and position
	8	Marked oil droplet deficiencies; severe prototrochal defects
	12	Oil droplet abnormalities; marked prototrochal deficiencies
	14	Dead
25	1	Prototrochal cell deficiencies; some pigment defects
	2	No pigment; prototrochal cell deficiencies; abnormalities in number and position of oil droplets
	4	Severe prototrochal deficiencies; no apical tuft. No anal pigment
	8	Prototrochal deficiencies; pigment abnormal. Oil droplet abnormalities in number and position
	12	Marked prototrochal cell deficiencies, no pigment, no apical tuft
	14	Dead
30	2	No pigment; marked prototrochal deficiencies. No apical tuft in most cases
	4	Dead
35	2	No pigment; marked prototrochal deficiencies. No apical tuft
40–90	1	Dead
50	½	No pigment; prototrochal deficiencies. Apical tuft usually absent
100	¼	Prototrochal and pigment deficiencies
	½	Dead

Treatment of the eggs with 15 per cent solutions for one to 4 hours produces pigment and prototrochal abnormalities, but after 6, 8, and 10 hours of treatment, the resulting larvae are marked by peculiar endodermal derangements which were at first thought to be the result of exogastrulation. However, closer examination of these cases revealed that the extruded cytoplasm was non-cellular insofar as could be determined, although it had the characteristic "glassy" appearance of endodermal cytoplasm; usually these larvae were marked by the complete absence of cilia (Fig. 5).

Frequently cases were observed in which the endoderm extended up under the surmounting cap of ectoderm. The cleavage pattern of the experimental eggs of this series showed no abnormality through the fourth cleavage. Repeated experiments with this treatment and with exposure of the eggs to concentrations of 12 per cent and 17 per cent for varying periods of time definitely established the fact that the abnormalities occur only after the treatment described above. It does not seem likely that such an extremely narrow range of dosage would be necessary for the production of true exogastrulae, inasmuch as these forms are produced in sea urchin eggs after a rather wide variety of treatments.

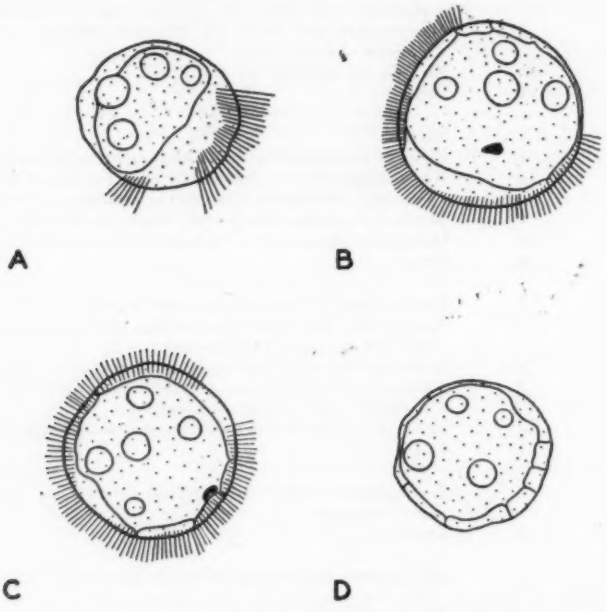

FIGURE 2. Typical 26–30 hour abnormal trochophores, showing anomalies in ciliary band, pigmentation and eye spots. Anal pigment and prototrochal pigment are absent in all cases; Figure 2A shows lengthening of the prototrochal cilia.

Exposure of the eggs to a 17 per cent solution resulted in generally abnormal trochophores after a treatment of one hour, and in more pronounced pigment and prototrochal abnormalities if treated for longer periods, up to the lethal point at 14 hours. No larvae with the endodermal derangements described above were noted in any of the cultures in this series. The effects of a 20 per cent solution for comparable periods of time approximate those described for a 17 per cent treatment; a concentration of 25 per cent produces pigment and prototrochal abnormalities after treatment for 1, 2, 4 and 8 hours. These defects also result from treatment for 2 hours with a 30 per cent solution. Concentrations of 40 per cent to 90 per cent

are lethal after treatment for one hour, and a 100 per cent solution has the same effect after ½ hour; the 100 per cent concentration produces pigment and prototrochal defects if applied for 15 minutes, so that its range of dosage is apparently very narrow. The reduction of the prototroch seems to increase quantitatively with an increase in the concentration of lithium and the duration of application.

Denuded Nereis eggs proved to be extremely sensitive to the effects of lithium, even in low concentrations for short periods of time. In all series, the controls showed considerable abnormality in shape and in the distribution of pigment, so that

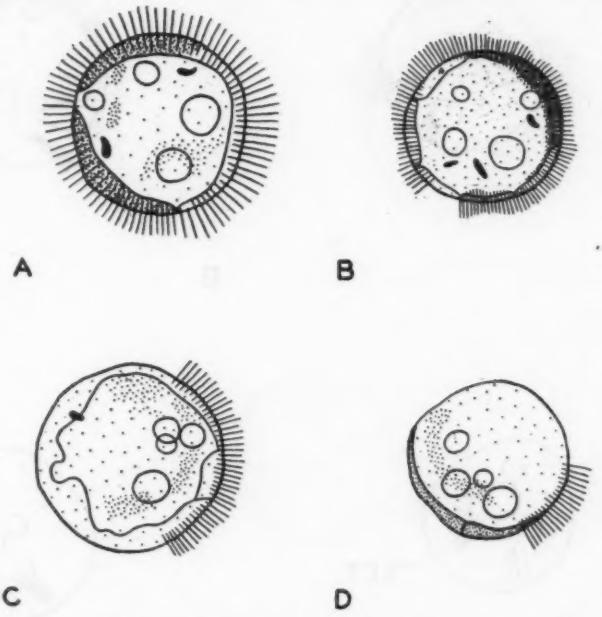

FIGURE 3. Abnormalities involving pigment, eye spots, cilia, and oil droplets after various treatments with lithium. Figure 3A is almost normal except for the heavy concentrations of anal and prototrochal pigment; Figure 3B indicates an abnormality in the number and position of the eye spots and in the size of these structures.

it is difficult to come to any definite decision as to the specific effects brought about by the lithium. However, in the cases in which membrane removal was not complete, the larvae survived fairly well, and in none of these cases were any indications of exogastrulation observed. After treatment for 2 hours with 15 per cent LiCl, some of the completely denuded larvae seemed to show signs of exogastrulation, although no conclusions can be drawn from this because of the small number of cases. Treatment of the denuded eggs with 5 per cent solutions for periods longer than 4 hours killed the eggs in late stages of cleavage, and no definite effects could be noted in larvae surviving in cultures which had been exposed for periods of 1 and 2 hours.

Discussion

From the foregoing results, it is apparent that the main effect of the lithium is to produce a reduction in the number of prototrochal cells, and in the quantity of anal and prototrochal pigments, together with effects on the eye spots and cilia. The absence of the apical tuft in most cases of prolonged or concentrated treatment indicates that the la-ld cells may be affected directly or indirectly, since it is known from the observations of Wilson (1892) that this quartet is associated with the formation

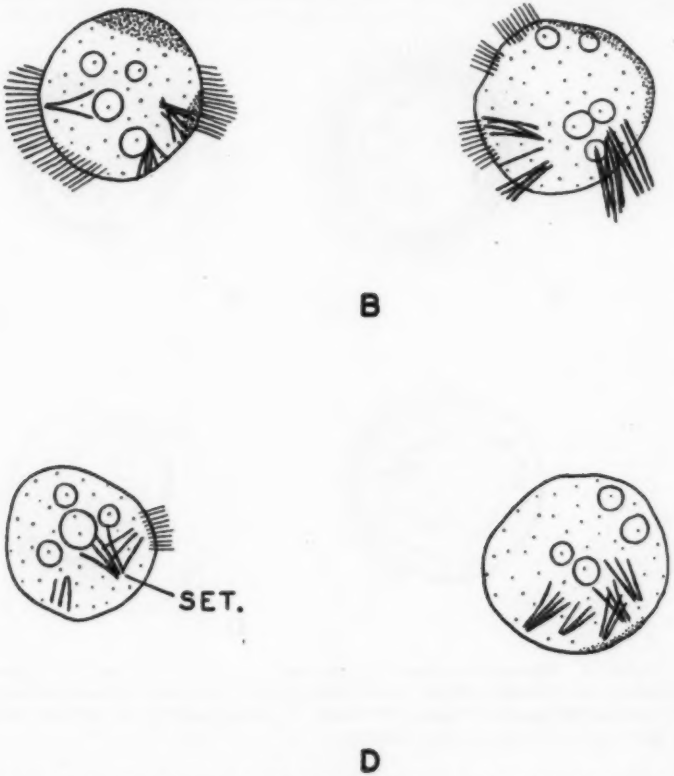

Figure 4. Marked abnormalities in setae of older larvae (ca. 53 hours). Figure 4B also shows an atypical number of oil droplets. Set. = Setae.

of the apical tuft. It is possible that the abnormalities observed in the seta sacs of older larvae (Fig. 4) may be due to the early action of the ion on the material destined to be incorporated into the 2d cell.

The absence of any true cases of exogastrulation in these experiments is quite striking. As was noted above, most of the forms in which this abnormality has been observed are marked by a type of gastrulation in which invagination plays a main

role. In the amphibian, formation of the endoderm is accomplished by a complex series of cell movements, as a result of which a directed and organized migration of cells brings about invagination of most of the vegetal hemisphere at the region of the blastopore. Meanwhile, the animal cells grow down to cover the vegetal region, thus forming the ectoderm of the embryo. The yolk material itself is invaginated in these forms and comes to be enclosed by the endoderm. Gastrulation in the echinoderms is likewise thought by Herbst (1893) to be brought about by an invaginative process. The strictly epibolic form of gastrulation exhibited by the egg of Nereis, on the other hand, is accomplished by a downgrowth of the ectomeres, so that even-

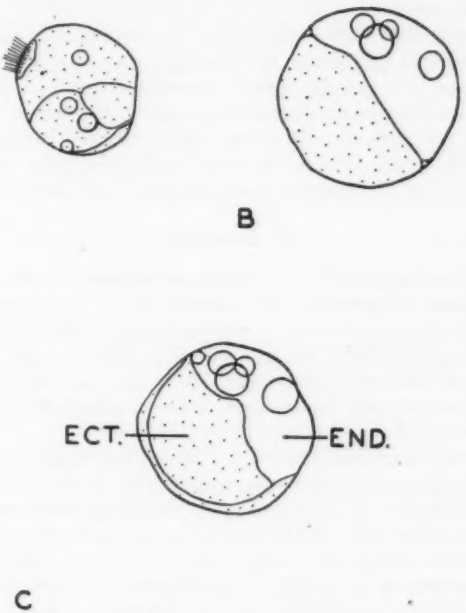

FIGURE 5. Abnormal trochophores resulting from treatment of eggs with 15 per cent LiCl for 6, 8 and 10 hours. Figure 5A shows no particular endodermal derangements, but has only one small tuft of cilia and is quite atypical in shape. Figures 5B and 5C show examples of "endodermal extrusions." Cell boundaries were not visible except as indicated. Ect. = Ectoderm; End. = Endoderm.

tually they cover the four entomeres. Such a movement seems to be associated with the presence of a "cellular affinity" as postulated by Costello (1940, 1945a) in connection with his experiments on the development of isolated blastomeres of this egg. Since no exogastrulation occurred, it may be assumed that this dynamic association of ectomeres and entomeres is not radically disturbed. However, the abnormalities in the number and position of the oil droplets in the four entomeres indicate that the lithium may exert some effect on the process of cytoplasmic segregation (Costello, 1945b) preceding the formation of the 3A, 3B, 3C and 4D cells.

No distinct line of demarcation can be drawn between the direct and the indirect effects of lithium in these experiments, since the ion is brought into contact with the egg before cleavage has occurred, and, in the case of the longer exposures, may remain until considerably after the completion of gastrulation. Runnström (1928b), observing lithium-treated sea urchin eggs under dark-field illumination, presented evidence that the element actually penetrates the cells. Spek (1918) maintained that the action of the lithium ion was brought about through its production of a precipitation and swelling effect on the surface of the vegetative cells. Thus, the exact mode of action remains obscure, but it appears fairly clear in the case of the egg of Nereis that exogastrulation is not produced, at least not in cases where the vitelline membrane is present.

The effects produced on the pigment of the trochophores seem to be at random, since the anal pigment may occur without the presence of the prototrochal pigment, and vice versa—or both may be present in varying degrees. This action possibly is related to the orientation of the eggs with respect to the bottom of the dish, or to each other; although no particular effort was made to keep the eggs suspended in the solution, the jelly serves to support them during the early stages of development, thus allowing relatively free access of the lithium to all surfaces.

Summary

1. The fertilized eggs of *Nereis limbata* were treated with mixtures of sea water and a 0.54 M stock solution of LiCl, ranging from 2 per cent to 100 per cent for periods of 15 minutes to 36 hours. Treatment was begun 75 minutes after insemination of the eggs, shortly before the appearance of the first cleavage.

2. No exogastrulae were observed. There were a few cases of marked abnormalities in the endodermal components of the trochophores within a very narrow range of treatment (15 per cent for 6, 8 and 10 hours).

3. The main abnormalities observed in the experimental larvae were: Absence of the apical tuft, lengthening of the prototrochal cilia, absence of these cilia in varying degrees, abnormalities in the anal and prototrochal pigments, absence or abnormality of the eye spots, deficiencies in the number of prototrochal cells, atypical seta sacs, abnormalities in the number and position of oil droplets. The degree of abnormality in these cases seemed to be roughly proportional to the severity of treatment.

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER THIGMOTRICHA). II. HYPOCOMIDES MYTILI CHATTON AND LWOFF, HYPOCOMIDES BOTULAE SP. NOV., HYPOCOMIDES PARVA SP. NOV., HYPOCOMIDES KELLIAE SP. NOV., AND INSIGNICOMA VENUSTA GEN. NOV., SP. NOV.

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Introduction

The genus *Hypocomides* was proposed by Chatton and Lwoff (1922a) to include two species: *Hypocomides mytili*, from *Mytilus edulis* L., and *H. modiolariae*, from *Modiolaria marmorata* (Forbes). These authors gave no formal diagnosis of the genus *Hypocomides*, however, and their incomplete descriptions of the two species are not supplemented by illustrations. They did not designate either species as the genotype. In 1926 Chatton and Lwoff published a preliminary diagnosis, again without illustrations, of a third species of *Hypocomides*, *H. zyrphaeae*, from *Zirfaea crispata* (L.). It was largely on the basis of their description of *H. zyrphaeae* that Raabe (1938) was led to suppose that one of the ciliates which he found parasitizing the gills of *Mytilus edulis* was *H. mytili*. He could not identify on this ciliate, however, a structure to which Chatton and Lwoff referred as the "vestige de frange adorale" and which according to them is in *H. mytili* "constitué seulement par une dizaine de grands cils." This "vestige de frange adorale" is supposed to be better developed in *H. modiolariae* than in *H. mytili*. In *H. zyrphaeae* it is represented, according to Chatton and Lwoff, by "une touffe de cils." The position of the "vestige de frange adorale" in relation to other ciliary structures in these three species of *Hypocomides* is entirely unclear.

On a ciliate which I have studied from *Mytilus edulis* from San Francisco Bay and which conforms in most respects to the description given by Raabe of the form considered by him to be *H. mytili*, I have been unable to detect such a "vestige de frange adorale." The brief notes on the morphology of *H. mytili* to be found in the papers of Chatton and Lwoff in which mention is made of this species (1922a,1922b, 1924) are not entirely consistent, and it is altogether possible that, as Raabe has pointed out, these authors used the term "vestige de frange adorale" to indicate only a short segment of the distal portion of one of the longer ciliary rows on the right side of the body, which Raabe suggested may be homologous with the two rows bordering the peristomal groove of ciliates of the family Ancistrumidae. It is quite evident from the brief description of *H. zyrphaeae*, however, that the "vestige de frange adorale" of this species is entirely separate from the two long rows on the right. Perhaps *H. mytili* and *H. modiolariae* are not actually congeneric with *H. zyrphaeae*, but this remains to be seen. At any rate, unless it can be established

with certainty that the ciliate described by Chatton and Lwoff as *Hypocomides mytili* is not identical with the species thought by Raabe to be *H. mytili*, it seems best to consider the form studied by Raabe to be *H. mytili* and to refer related forms to the same genus.

In the present paper I will give a description of the ciliate from *Mytilus edulis* which I consider to be *H. mytili*, and will add three new species to the genus *Hypocomides: H. botulae* sp. nov. and *H. parva* sp. nov., from the gills and palps of the rock-boring pelecypod, *Botula californiensis* (Philippi), and *H. kelliae* sp. nov., from *Kellia laperousii* Deshayes, a small nestling clam which is frequently encountered in the excavations made in rocks by other molluscs. Another very interesting ancistrocomid ciliate from *Botula californiensis* will be described herein as *Insignicoma venusta* gen. nov., sp. nov.

Hypocomides mytili Chatton and Lwoff

(Fig. 1; Plate I, Fig. 1)

The body is elongated and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior three-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually not quite so rounded as the right margin, and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Thirty living individuals taken at random ranged in length from 34 μ to 48 μ, in width from 16 μ to 22 μ, and in thickness from 13 μ to 18 μ, averaging about 40 μ by 18 μ by 14.5 μ.

The anterior end of the body is provided with a short contractile tentacle which enables the ciliate to attach itself to the epithelial cells of the gills and palps of the host and to suck out their contents. This tentacle is continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for about one-half the length of the body. The canal nearly always appears to be widest in its anterior portion and to be directed obliquely toward the right side of the body as it extends posteriorly.

The cilia of *H. mytili* are about 9 μ in length and are markedly thigmotactic, especially near the anterior end of the body. The ciliary system consists of three separate complexes. The central complex, occupying the middle and right portions of the ventral anterior concavity, consists of seven rows, the one nearest the right being the shortest (one-third to two-fifths the length of the body), the other rows becoming progressively longer toward the left. The sixth and seventh rows are usually approximately one-half the length of the body; in some specimens the seventh row is appreciably shorter than the sixth. To the right of the central complex are two long rows, each about one-half the length of the body. Both these rows originate on the dorsal surface close to the left margin a short distance behind the level of origin of the rows of the central complex and curve ventrally as they extend posteriorly. To the left of the central complex is a series of eight rows which usually are more closely-set than those making up the central complex. The innermost row, which originates on the left margin of the body near the base of the suctorial tentacle, is the shortest, and terminates at a point about one-third the distance from the anterior end of the body to the posterior end. The remaining rows become pro-

gressively longer and originate progressively more dorsally and posteriorly, curving ventrally as they extend backward. The outer row of this complex is usually the longest and terminates at a point nearly opposite the point of termination of the outer of the two rows constituting the complex on the right.

According to Raabe, the three ciliary complexes of *H. mytili* are much more distinctly separated than I have observed them to be. Raabe also stated that variations in the number of ciliary rows in the central and left complexes are correlated with two well-differentiated size races: form *minor* (17 μ to 26 μ in length) and form *major* (26 μ to 36 μ in length). I have noted no tendency for the ciliates I consider to belong to this species to be segregated into distinct size races and have seen no examples of *H. mytili* which were in life as small as those assigned to the form *minor* by Raabe. Although I have observed few deviations from the typical number of ciliary rows, it may be of interest to record here the fact that frequently some

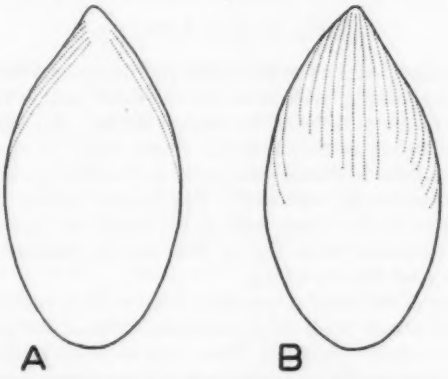

FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Distribution of ciliary rows, somewhat diagrammatic.[1] A, dorsal aspect; B, ventral aspect.

of the rows of the left complex, particularly the first and second rows, do not stain as well with hematoxylin or impregnate as well with activated silver albumose (protargol) as do the rows of the central and right complexes, and hence may easily escape detection.

The cytoplasm is colorless and contains numerous lipoid droplets in addition to food inclusions. Several larger food vacuoles are sometimes observed in the posterior part of the body. The contractile vacuole is centrally located[2] and opens to the exterior on the ventral surface. I have observed no permanent opening in the pellicle.

The macronucleus is usually ovoid, sometimes sausage-shaped; more rarely it is spherical, although Raabe considered the macronucleus of *H. mytili* to be typically

[1] All text figures illustrating this paper are based on camera lucida drawings of individuals fixed in Schaudinn's fluid and impregnated with activated protein silver (protargol).

[2] Raabe stated that the contractile vacuole of *H. mytili* is to be found in a vacuolated area in the posterior part of the body behind the macronucleus. Perhaps his observation was based on specimens which were undergoing degenerative vacuolization and in which the contractile vacuole was not conspicuous. In all normal individuals which I have examined its position was central.

spherical. It is situated dorsally in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from 9 μ to 13.2 μ and in width from 4.4 μ to 6.9 μ.

The spherical micronucleus is ordinarily situated near the middle of the body anterior to the macronucleus, although sometimes it is seen to lie to one side of the macronucleus. The chromatin appears in most fixed and stained specimens to be homogeneous, although in some it is aggregated into vague peripheral granules or strands. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.7 μ to 4 μ.

I found *Hypocomides mytili* to be present in large numbers on the gills and palps of about 80 per cent of the specimens of *Mytilus edulis* which I examined from various localities in San Francisco Bay. It is sometimes the only ciliate infesting the mussels, but more commonly it is associated with *Crebricoma carinata* (Raabe) and *Ancistruma mytili* (Quennerstedt).

Hypocomides mytili Chatton and Lwoff

Diagnosis: Length 34 μ–48 μ (according to Raabe 17 μ to 36 μ), average about 40 μ; width 16 μ–22 μ, average about 18 μ; thickness 13 μ–18 μ, average about 14.5 μ. The central ciliary complex is composed of seven rows (according to Raabe seven or eight rows) which are one-third to one-half the length of the body, becoming progressively longer toward the left side; the right complex consists of two rows, each about one-half the length of the body; the left complex consists of eight closely-set rows (according to Raabe five [?] or six rows) one-third to one-half the length of the body. The contractile vacuole is central and opens to the exterior on the ventral surface. The macronucleus is typically ovoid or sausage-shaped, rarely spherical. The micronucleus is spherical. Parasitic on the epithelium of the gills and palps of *Mytilus edulis* L. (Roscoff [Chatton and Lwoff]; Hel [Raabe]; San Francisco Bay, California).

HYPOCOMIDES BOTULAE SP. NOV.

(Fig. 2; Plate 1, Fig. 2)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually less rounded than the right margin and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from 31 μ to 39 μ, in width from 14 μ to 17 μ, and in thickness from 12 μ to 14 μ, averaging about 33 μ by 15 μ by 13 μ.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for a distance equal to about three-fifths the length of the body.

In most individuals the canal appears to be directed obliquely toward the right side of the body.

The cilia are about 8 μ to 9 μ in length and are markedly thigmotactic, particularly near the base of the suctorial tentacle. The ciliary system consists of three separate complexes. The central complex, occupying the larger part of the ventral anterior concavity, consists of eleven rows about one-half the length of the body which become progressively longer toward the left side. To the right of the central complex are two longer rows which originate on the dorsal surface close to the right margin a little behind the level of origin of the rows of the central complex and curve downward as they extend posteriorly. Each of the two rows is about three-fifths the length of the body. To the left of the central complex is a series of eleven rather closely-set rows which originate on the left lateral margin and the dorsal surface on the left side and curve ventrally as they extend posteriorly. These

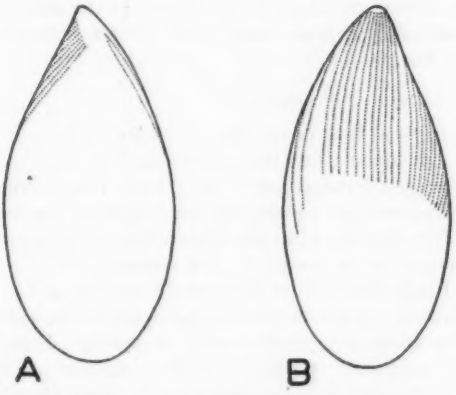

FIGURE 2. *Hypocomides botulae* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

rows are about one-half the length of the body and originate and terminate progressively more posteriorly.

The cytoplasm is colorless and contains numerous lipoid droplets in addition to food inclusions. I have not seen any large food vacuoles in this species. The contractile vacuole, situated near the middle of the body, opens to the exterior on the ventral surface.

The macronucleus is ovoid to sausage-shaped and is situated in the posterior half of the body. Its longitudinal axis is usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin is aggregated into a dense reticulum. Vacuole-like clear spaces of varying size are sometimes apparent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 9 μ to 13 μ and in width from 4.3 μ to 7 μ.

The micronucleus is spherical and is situated dorsally near the middle of the body. The chromatin appears to be homogeneous in fixed specimens. In ten individuals

fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.4 μ to 3.2 μ.

Hypocomides botulae was present in small numbers on the gills and palps of twelve of the thirty-four specimens of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes found in association with *Hypocomides parva* and *Insignicoma venusta*. No specimens of *Botula falcata* (Gould) which I examined from the same localities were parasitized by these or any other ciliates.

Hypocomides botulae sp. nov.

Diagnosis: Length 31 μ–39 μ, average about 33 μ; width 14 μ–17 μ, average about 15 μ; thickness 12 μ–14 μ, average about 13 μ. The central ciliary complex consists of eleven rows each about one-half the length of the body; the right complex is composed of two rows about three-fifths the length of the body; the left complex consists of eleven closely-set rows about one-half the length of the body. The macronucleus is ovoid to sausage-shaped. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

Hypocomides parva sp. nov.

(Fig. 3; Plate I, Fig. 3)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin, and appears typically to be nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty-five living individuals taken at random ranged in length from 21 μ to 29 μ, in width from 10 μ to 13 μ, and in thickness from 8 μ to 11 μ, averaging about 26 μ by 12 μ by 10 μ.

The anterior end of the body is provided with a short contractile suctorial tentacle continuous with an internal tubular canal. The canal is usually directed obliquely toward the right side and can be traced in most fixed specimens stained with iron hematoxylin for about one-half the length of the body.

The cilia of *Hypocomides parva* are about 6 μ to 7 μ long and are strongly thigmotactic, particularly near the base of the suctorial tentacle. The ciliary system consists of three separate complexes. The central complex, which occupies the larger part of the concave depression on the anterior part of the ventral surface, consists of eight rows which are about two-fifths the length of the body. To the right of this complex are two longer rows which originate on the dorsal surface close to the right margin behind the level of origin of the rows that constitute the central complex. Each of these rows is about three-fifths the length of the body and curves ventrally as it extends posteriorly. The outer row originates and terminates the more posteriorly. To the left of the central complex is a series of eight rows which originate on the left lateral margin and on the left side of the dorsal surface and curve ventrally as they extend backward. These rows are about two-fifths the length of the body and originate and terminate progressively more posteriorly. The outermost

row, however, is somewhat shorter than the other rows and is usually seen to terminate a little anterior to the point of termination of the seventh row.

The cytoplasm is colorless and contains numerous small lipoid droplets and food inclusions. I have distinguished no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is typically ovoid, sometimes spherical, rarely sausage-shaped. It is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing a few vacuole-like clear spaces of varying size. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 4.2 μ to 8.2 μ and in width from 4.2 μ to 5.3 μ.

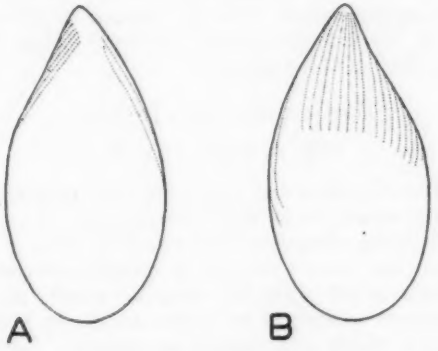

FIGURE 3. *Hypocomides parva* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

The micronucleus is usually spherical and is situated dorsally a short distance anterior to or to one side of the macronucleus. Sometimes it is located as far anteriorly as the middle of the body. In fixed and stained preparations the chromatin of the micronucleus appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 1.9 μ to 2.3 μ.

Hypocomides parva was present on the gills and palps of nineteen of the thirty-four individuals of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes associated with *H. botulae* and *Insignicoma venusta*. In my experience it is the most common species of ciliate parasitizing this mollusc.

Hypocomides parva sp. nov.

Diagnosis: Length 21 μ–29 μ, average about 26 μ; width 10 μ–13 μ, average about 12 μ; thickness 8 μ–11 μ, average about 10 μ. The central ciliary complex comprises eight approximately equal rows about two-fifths the length of the body;

the right complex consists of two rows about three-fifths the length of the body; the left complex consists of eight rows, each about two-fifths the length of the body, the eighth row being usually somewhat shorter than the others. The macronucleus is typically ovoid. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMIDES KELLIAE SP. NOV.

(Fig. 4; Plate I, Fig. 4)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-third of the ventral sur-

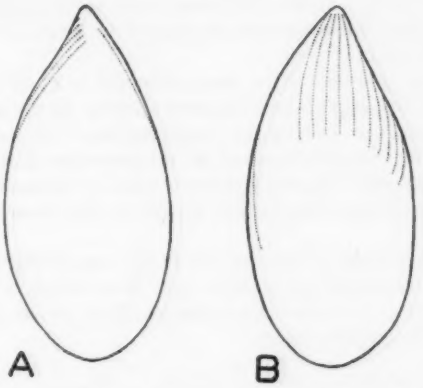

FIGURE 4. *Hypocomides kelliae* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

face; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is not as rounded as the right margin, and typically is nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from 31 μ to 37 μ, in width from 13 μ to 15 μ, and in thickness from 11 μ to 13 μ, averaging about 33 μ by 14 μ by 12 μ.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The canal is usually directed toward the right side of the body as it extends posteriorly. It can be traced in most fixed individuals stained with iron hematoxylin for about three-fifths the length of the body.

The thigmotactic cilia of *H. kelliae* are about 8 μ or 9 μ in length. The ciliary system is composed of three separate complexes. The central complex, occupying the larger part of the anterior ventral depression, consists of five equal rows about one-third the length of the body. To the right of this system is a single long row about two-thirds the length of the body. This row originates on the dorsal surface

close to the right margin a little behind the level of origin of the central rows and curves ventrally as it extends posteriorly. To the left of the central complex is a series of five rows, one-third the length of the body, which originate on the left lateral margin or dorsal surface on the left side and curve ventrally as they extend posteriorly. These rows originate and terminate progressively more posteriorly.

The cytoplasm is colorless and contains numerous small lipoid droplets and food inclusions. I have observed no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped, usually about two times as long as wide. It is situated in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be organized into a very dense reticulum which sometimes is seen to enclose vacuole-like clear spaces of varying size. These are more evident near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 7.8 μ to 14 μ and in width from 3.9 μ to 7 μ.

The micronucleus varies in shape from spherical to ovoid; typically it is ovoid. It is usually situated dorsally a short distance anterior to the middle of the body or to one side of the anterior part of the macronucleus. In most fixed and stained individuals of *H. kelliae* the chromatin of the micronucleus is aggregated into deeply-staining peripheral strands. In ten specimens fixed in Schaudinn's fluid and stained by the Feulgen reaction the micronucleus ranged in size from 1.9 μ by 1.5 μ to 2.3 μ by 1.9 μ.

Hypocomides kelliae was present in nine of the twenty-eight individuals of *Kellia laperousii* which I examined from localities near Moss Beach, California. Also associated with this mollusc is a small ancistrumid ciliate which it may be possible for me to describe in a subsequent paper.

Hypocomides kelliae sp. nov.

Diagnosis: Length 31 μ–37 μ, average about 33 μ; width 13 μ–15 μ, average about 14 μ; thickness 11 μ–13 μ, average about 12 μ. The central ciliary complex consists of five rows about one-third the length of the body; the right complex consists of a single row about two-thirds the length of the body; the left complex consists of five rows about one-third the length of the body. The macronucleus is ovoid or sausage-shaped. The micronucleus is typically ovoid. Parasitic on the epithelium of the gills and palps of *Kellia laperousii* Deshayes (Moss Beach, California). Syntypes are in the collection of the author.

INSIGNICOMA VENUSTA GEN. NOV., SP. NOV.

(Fig. 5; Plate I, Fig. 5)

The body is elongated, narrowed anteriorly, and somewhat flattened dorsoventrally. The anterior one-half of the ventral surface, on which the major part of the ciliary system is disposed, is weakly concave; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin and typically is nearly straight or slightly indented. The body is widest near the middle and rounded poste-

riorly. Twenty-five living individuals taken at random ranged in length from 42 μ to 52 μ, in width from 18 μ to 21 μ, and in thickness from 15 μ to 18 μ, averaging about 48 μ by 20 μ by 17 μ.

The anterior end of the body is provided with a contractile suctorial tentacle continuous with an internal tubular canal. In most fixed specimens stained with iron hematoxylin the canal can be traced for about three-fifths the length of the body. It usually appears to be directed obliquely toward the right side.

The ciliary system consists of four separate complexes. Two long, widely-spaced rows on the right side of the body originate on the dorsal surface close to the right margin at the anterior end and are about two-thirds the length of the body. They curve ventrally as they extend posteriorly. A central complex of fourteen or fifteen

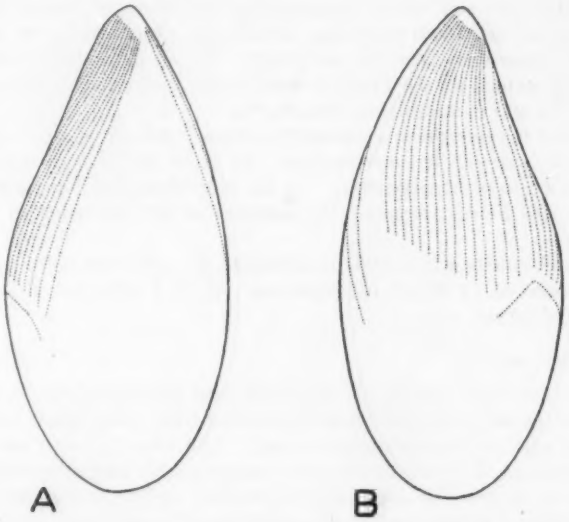

FIGURE 5. *Insignicoma venusta* gen. nov., sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

rows occupies the larger part of the ventral anterior depression. These rows are on the average about one-half the length of the body and originate progressively more posteriorly toward the left side. The outer two or three rows on the left, however, do not usually terminate quite so far posteriorly as the twelfth row. The rows of this complex are usually a little more closely-set on the right side than on the left. To the left of the central complex is a series of sixteen or seventeen rows about one-half the length of the body which with the exception of the outer three or four rows are very closely-set. The innermost row originates on the left lateral margin near the base of the suctorial tentacle; the remaining rows originate progressively more dorsally and posteriorly. The distal portions of several of the inner rows of this complex are usually visible on the left side of the ventral surface. Posterior to the middle of the body on the left side is a nearly V-shaped series of cilia which originates

on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of this fourth complex are about 12 μ to 14 μ in length. The cilia of the other rows are about 8 μ or 9 μ in length and strongly thigmotactic, especially near the base of the suctorial tentacle.

The cytoplasm is colorless and contains numerous small lipoid droplets in addition to food inclusions. A few larger food vacuoles are sometimes observed near the posterior end. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped and is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying sizes. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 12 μ to 17 μ and in width from 4.4 μ to 9 μ.

The spherical micronucleus is commonly situated dorsally a short distance anterior to or to one side of the macronucleus. In fixed and stained preparations the chromatin appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from 2.4 μ to 4 μ.

Insignicoma venusta was found to parasitize the gills and palps of nine of the thirty-four specimens of *Botula californiensis* which I collected at localities near Moss Beach, California.

Insignicoma gen. nov.

Diagnosis: The body is elongated and somewhat flattened dorso-ventrally. The anterior end of the body is narrowed and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary system consists of four separate complexes. The central complex, occupying the major portion of the shallow concavity on the anterior one-half of the ventral surface, is bounded on the right by a small number of widely-spaced rows which curve ventrally as they extend posteriorly; to the left of the central complex is a series of closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left lateral margin and dorsal surface on the left side and curve ventrally as they extend backward; the fourth complex consists of a V-shaped series of long cilia which lies immediately behind the distal portion of the gap separating the central and left ciliary complexes. The contractile vacuole is central and opens to the exterior

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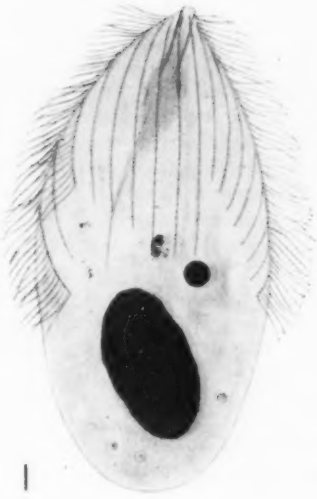
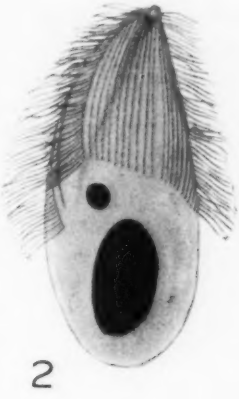
PLATE I

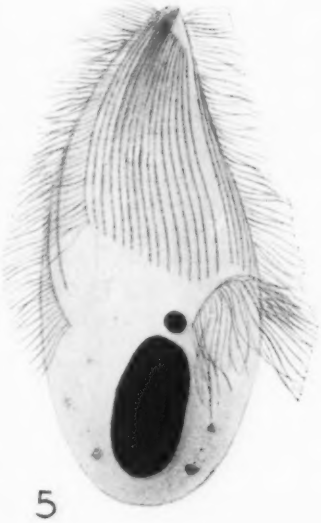
All figures have been drawn with the aid of a camera lucida from specimens fixed in Schaudinn's fluid and stained with iron hematoxylin. × 1870.

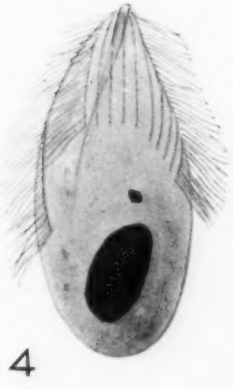
FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Ventral aspect.
FIGURE 2. *Hypocomides botulae* sp. nov. Ventral aspect.
FIGURE 3. *Hypocomides parva* sp. nov. Ventral aspect.
FIGURE 4. *Hypocomides kelliae* sp. nov. Ventral aspect.
FIGURE 5. *Insignicoma venusta* gen. nov., sp. nov. Ventral aspect.

PLATE I









on the ventral surface; there is no permanent opening in the pellicle. Genotype: *Insignicoma venusta* gen. nov., sp. nov.

Insignicoma venusta gen. nov., sp. nov.

Diagnosis: Length 42 μ–52 μ, average about 48 μ; width 18 μ–21 μ, average about 20 μ; thickness 15 μ–18 μ, average about 17 μ. The central ciliary complex consists of fifteen (rarely fourteen) rows about one-half the length of the body which originate progressively more posteriorly toward the left side; the right complex consists of two widely-spaced rows about two-thirds the length of the body which originate on the dorsal surface close to the left margin and curve ventrally as they extend posteriorly; the left complex consists of sixteen or seventeen closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left margin and dorsal surface on the left side and curve ventrally as they extend posteriorly; the V-shaped series of cilia constituting the fourth complex originates on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of the fourth complex are approximately 12 μ–14 μ in length; those of the other three complexes are approximately 8 μ–9 μ in length. The macronucleus is ovoid or sausage-shaped; the micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

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NATURAL HETEROAGGLUTININS IN THE BODY-FLUIDS AND SEMINAL FLUIDS OF VARIOUS INVERTEBRATES[1]

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Introduction

Since the early work of Landois (1875) numerous investigators have noted in the normal serum of various species of animals, particularly among the vertebrates, the occurrence of agglutinins that act on the cells of certain other species (cf. Wiener, 1943; Landsteiner, 1945; Thomsen, 1932). Such natural heteroagglutinins have also been frequently reported to occur in the serum or body-fluids of various invertebrates (see literature in Huff, 1940; Tyler and Metz, 1945). Heteroagglutination reactions are frequently encountered in fertilizin studies and have been reported by several investigators (Lillie, 1913, 1919; Glaser, 1914; Just, 1919, 1930; Sampson, 1922; Godlewski, 1934; Hartmann *et al.*, 1940; Runnström *et al.*, 1944) as occurring between spermatozoa and foreign egg-water preparations, body-fluids and spermatozoa or their extracts. Further information concerning the range and nature of the heteroagglutination reactions is of importance, then, in analysis of problems of fertilization particularly in regard to the specificity and role of the interacting substances that are obtained from eggs and sperm.

A study of heteroagglutination reactions with lobster serum (Tyler and Metz, 1945; Tyler and Scheer, 1945), which normally acts on the sperm or the blood-cells of a wide variety of species throughout the animal kingdom, showed that at least ten distinct relatively class-specific agglutinins are present in the serum of this species. This was determined by means of absorption tests. In the controls for those tests the supernatant fluids of the sperm suspensions used for absorption were also examined for agglutinating activity and it was found, particularly when the sperm had not been previously washed, that the fluids from sperm of some species were active on cells of certain other species. Tests were then made with the body-fluids and these, too, were found to be active.

The present paper reports the results of this examination of the body-fluids and the sperm-supernatants of various species of invertebrates for the occurrence of heteroagglutinins. The species examined were for the most part those that have been used or are potentially useful in fertilizin studies. In addition a few absorption tests were made with starfish body-fluid to determine whether or not its agglutinating activity is attributable to the presence of several heteroagglutinins, each with broad specificity such as was found in lobster serum.

Materials and Methods

The body-fluids of twelve species of animals among the annelids, echinoderms, mollusks and tunicates were examined for possible agglutinating action on sperm

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suspensions of various species of invertebrates and, in a few cases, on erythrocyte suspensions of various vertebrates. The body-fluids were obtained by incision into the body cavity or by insertion of a hypodermic syringe into the body cavity. In the case of Ciona the fluid was obtained directly from the heart. The fluids were clarified by centrifugation and tested in the manner previously described (Tyler and Metz, 1945). The supernatant fluids of sperm suspensions of eleven of the same species were also tested. These were obtained by centrifugation of approximately 10 to 20 per cent suspensions of "dry" sperm in sea water. They may be termed diluted seminal fluids.

Experimental Part

In all of the species that were examined the body-fluids were found to possess agglutinating activity for the cells of certain other species. The results are presented in Table I. In some species (e.g., 1, 6, 18, 19, 21) the fluids exhibited agglutinating action on the cells of most of the species that were tested. However, the fluids of

Table I

Heteroagglutinating action of body-fluid (b) and seminal fluid (s) of various invertebrates

Spermatozoa (species 1 to 25) or erythrocytes (species 26–34) of:	Fluids of:																							
	1		4		6	12		13		14		16		18		19		21		22		23	
	b	s	b	s	b	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s
POLYCHAETS																							
1. *Chaetopterus variopedatus*	0	0	0	0	+	0	0	+	+	+	+	+	+	+	+	+	+	+		0	0	0	0
2. *Halosydna johnsoni*		0	0	0	+	0	0	+	+	+	+			+	+								
3. *Sabellaria californica*	+	+	0	0		0	0	0	0	0	0	0		+		+	+	0				0	0
ECHIUROIDS																							
4. *Urechis caupo*	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+		+		+	
5. *Thalassema* sp.	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+		+			
AMPHINEURANS																							
6. *Mopalia muscosa*	+	+	0	0	0	+	0	+	+	0	0	0	0	+	+		+	0		+		+	+
7. *Ischnochiton magdalensis*	+	+	0	0	0	+		+	+	0	0	0		+	+		+	0		+			+
GASTROPODS																							
8. *Acmea digitalis*	+	+	+		0	0				0	0			+	0			0				+	+
9. *Lottia gigantea*	+	+	+	0	0	0	0	+		0				+				0		+			
10. *Tegula galena*	+	+	+		+	0					0			+	+			+		+			
11. *Astraea undosa*	+	+			+	0		+	0	0				+	+			+		+			+
12. *Megathura crenulata*	+	+	+	0	+	0	0	+	+	0	0	0	0	+	+	+		+		+		+	+
PELECYPOD																							
13. *Mytilus californianus*	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+		+		+		+	
ECHINOIDS																							
14. *Strongylocentrotus purpuratus*	0	0	0	0	+	0	0	+	+	0	0	0	0	0	0	0	0	+	0		0	0	0
15. *S. franciscanus*					+	0		+			0	0	0	0	0								
16. *Lytechinus pictus*	0	0	0	0	+	0	0	+	+	0	0	0	0	0	0	0		+		0		0	
17. *Dendraster excentricus*		0	0	0	+	0			+	0	0		0	0	0					0			

TABLE I—*Continued*

Spermatozoa (species 1 to 25) or erythrocytes (species 26–34) of:	Fluids of:																					
	1		4		6	12		13		14		16		18		19		21		22		23	
	b	s	b	s	b	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s	b	s
ASTEROIDS																							
18. *Patiria miniata*	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0	0		+		0	0	0	0
19. *Pisaster ochraceus*	+	+	0	0	0	+	+	0	0	0	0	0	0	0	0	0	0	+		0	0		0
20. *Astropecten armatus*		+		0					0	0	0	0	0	0	0								
HOLOTHURIOID																							
21. *Stichopus californicus*				0			0		0		0		0		0	0	0		0	0			
ASCIDIANS																							
22. *Ciona intestinalis*	+	+	0	0	+	0	0	0	0	0	0	0	0	+	+	+	+	+		0	0	0	0
23. *Styela barnharti*	+	+	0	0	+	0		0	0	0	0	0	0	+	+	+	+	+	+	0	0	0	0
24. *Ascidia ceratodes*		+	0	0		0		0		0				+	+			+		0	0	0	0
FISH																							
25. *Leuresthes tenuis*	0		0			0		0		0	0		0	0	0			+	+		+		
26. *Girella nigricans*	0								0	0	0		0	0	0		0	+	+				
AMPHIBIA																							
27. *Rana catesbiana*	+	+																					
28. *Bufo halophilus*		+		0		0			0		0				0		0		0		+		+
REPTILE																							
29. *Sceloporus occidentalis*	+	0									0	0			0	0		+	+		0		
BIRD																							
30. Chicken		+		0			0		+		0			+	0		0		+		0		0
MAMMALS																							
31. Guinea pig								+	+	0				+									
32. Rabbit									+		0												
33. Sheep		+		0			0	+	+		0		0	+							0		0
34. Man									+		0			+									

none of the twelve species examined were found to possess agglutinating action on all of the species that were tested.

With one exception no heteroagglutination reactions occurred with body-fluids and cells of animals belonging to the same taxonomic class. The exception to this consists in the agglutination of Sabellaria sperm by Chaetopterus fluid. It is of interest in this connection that these two genera are placed (Pearse, 1936) in separate subclasses (cryptocephala and phanerocephala respectively) of the polychaets. Sabellaria also differs from the other two polychaets that were tested in that its cells fail to agglutinate in the fluid of the mussel (13), sea-urchins (14, 16) and sea cucumber (21) as well as that of the lobster previously (Tyler and Metz, 1945) reported.

Another feature of the results is that closely related species behave alike with respect to the ability or inability of their cells to be agglutinated by the various body-fluids. This was evident in the previously reported experiments with lobster serum.

In the present data differences are observed between species belonging to different orders, as in the case of the two species (8 and 9) belonging to the docoglossid gastropods which differ from the three rhipidoglossid species (10, 11, and 12) in their reactions to two of the fluids (6 and 21). With the other twenty-nine species tested, similarity in behavior is exhibited by members of the same class or sub-class.

The tests with the diluted seminal fluids gave results that in most cases paralleled those obtained with the body-fluids. Eight exceptions (1 on 29; 4 on 9 and 12; 12 on 6; 13 on 11; 18 on 8 and 30; 21 on 14) may be noted in Table I out of a total of 186 cross-combinations in which both seminal fluid and body-fluid were examined. These exceptions are all in the same direction; namely, a failure of the dilute seminal fluid to cause agglutination while the corresponding body-fluid is active. It can, then, be stated that in those cases in which the diluted seminal fluid is found to possess heteroagglutinating activity, the corresponding body fluid is likewise found to be active. Thus, similarly acting heteroagglutinins are found in both body-fluid and seminal fluid. The above-mentioned few exceptions may be attributed to failure to obtain, in certain seminal fluid preparations, sufficient concentration of a particular heteroagglutinin to produce a visible reaction with the cells of some species. However, the tests necessary to determine the validity of this explanation have not, as yet, been made.

From the similarity in action of seminal fluid and body-fluid, it might be inferred that the activity of the former is due to contamination with the latter. However, it may be noted that in most of the species employed for preparation of seminal fluid (e.g., 4, 12, 14, 16, 18, 19, 21, 22) the sperm is readily obtained without any appreciable admixture of body-fluid. Another interpretation is that seminal fluid is normally similar to body-fluid in composition. Against this may be cited the fact that readily recognizable constituents of body-fluid, such as hemocyanin in the mollusks, are not observed in the seminal fluids. A third possibility is that identical heteroagglutinins are present in both fluids. However, serological similarity does not imply entirely identical molecular constitution. Reaction with a specific antigen implies similarity only on the part of the specific combining groups of the antibodies from diverse sources. In the present case it has not been shown that the heteroagglutinin in seminal fluid and that in body-fluid both react with the same antigenic group or structure on the sperm that they agglutinate. However, the generally parallel behavior of the two fluids, when tested with spermatozoa of different species, favors that view.

Absorption tests, to determine whether or not more than one heteroagglutinin is involved in the action of a particular fluid, were carried out with Patiria body-fluid. These were done in the manner previously described (Tyler and Metz, 1945). Before being used for absorption the sperm were washed repeatedly in order to free them of agglutinins contributed by the seminal fluid, and this was checked in each test by examination of the supernatant of an aliquot part of the sperm for agglutinating activity.

Samples of Patiria body-fluid were absorbed with sperm of six species of animals and tested for agglutinating activity on sperm of nine species. The results are given in Table II. This limited set of tests reveals the presence of at least four distinct heteroagglutinins in Patiria body-fluid. These evidently comprise:—one for the two polychaets, one for the two echiuroids and Mytilus, one for the two gastropods, and one for the two ascidians. It seems likely, then, that the situation in Patiria body-

fluid is similar to that previously reported for the lobster; namely, the presence of a number of distinct agglutinins, each with broad group specificity.

Discussion

Heteroagglutinins are evidently normal constituents of the body-fluids of animals. They have generally been considered to be non-specific agents. However, from the fact that the fluids of various species act on the cells of different assemblages of other species, the heteroagglutinins must be regarded as having some degree of specificity. The previously reported (Tyler and Metz, 1945) absorption tests with lobster-serum gave evidence of the presence of ten distinct heteroagglutinins which are, for the most part, each specific for a taxonomic class of animals. The present results with Patiria body-fluids are indicative of similarly broad specificity on the part of the four heteroagglutinins found therein. It is clear, however, that heteroagglutinins of different species and also different heteroagglutinins of the same animal may differ considerably in the range of species on which they act. The rule is

Table II

Agglutinative activity of Patiria body-fluid after absorption with spermatozoa of various species

Spermatozoa of:	Body-fluid absorbed with washed sperm of:					
	Chaetopterus	Urechis	Thalassema	Astraea	Megathura	Mytilus
Chaetopterus	0	+	+	+	+	+
Halosydna	0	+	+	+	+	+
Urechis	+	0	0	+	+	0
Thalassema	+	0	0	+	+	0
Astraea	+	+	+	0	0	+
Megathura	+	+	+	0	0	+
Mytilus	+	0	0	+	+	0
Ciona	+	0	0	+	+	+
Ascidia	+	0	0	+	+	+

that closely related species react alike to a particular fluid, but the closeness of relationship required depends upon the particular heteroagglutinating fluid employed. In the present tests (Table I) we find that species that belong to the same class, in most instances, behave alike with respect to the ability or inability of their cells to be agglutinated by the body-fluids of all twelve of the species examined.

In the sera of various mammals natural heteroagglutinins are found (cf. Thomsen, 1932; Wiener, 1943; Landsteiner, 1945) that are relatively species-specific and in human sera, as is well known, natural isoagglutinins are encountered that differentiate groups of individuals. In the various invertebrate body-fluids examined, agglutinins of such specificity have not, as yet, been found, the fluid of a particular species being inactive on cells of closely related species. However, from the ripe gametes, of many of these species natural agglutinins are obtained that act within the species. These consist in the fertilizins from eggs and antifertilizins from sperm described by Lillie (1913 et seq.), Just (1930), Frank (1939), Tyler (1939 et seq.), Hartman *et al.* (1939 et seq.), Runnström *et al.* (1942 et seq.) and others. While these agents act on gametes of the opposite sex within the species, they also have

been found to act on closely related species and in some instances the preparations act on remotely related species. Thus Arbacia egg water was found (Lillie, 1913) to agglutinate Nereis sperm, and sea-urchin eggs have been found (Runnström *et al.*, 1942) to be agglutinated by sperm extracts of animals as remotely related as the salmon and the ox. In Lillie's experiment it was shown that absorption with Nereis sperm removed the cross-reacting substance from Arbacia egg water without diminishing its agglutinating action on Arbacia sperm. Similar absorption experiments have not been reported in most of the cross-heteroagglutination reactions obtained by later workers with fertilizin and antifertilizin preparations. The need for such experiments is quite evident before any adequate determinations can be made of the specificity of these interacting substances obtained from the gametes. The present results may provide a helpful basis of procedure in such experiments.

The bearing of the heteroagglutination reactions on phylogenetic questions has been previously (Tyler and Metz, 1945) discussed. The present results are consistent with the previously expressed view that the reactivity of the cells of a particular species with various fluids is a characteristic of a group of related species and constitutes a group-specific trait in addition to the various group-specific morphological and chemical features of animals. There is no reason, as yet, for considering this trait to be of more general significance than any other in any applications that might be made to phylogenetic problems.

Summary

1. The body-fluids of 12 species of invertebrates (including two ascidians) and the seminal fluids of 11 species were examined for agglutinating action on the spermatozoa or blood cells of 34 species of animals.

2. All of the fluids were found to contain agglutinins for the cells of some of the species tested. Five of the fluids gave reactions with most of the species but none reacted with all of the species.

3. With one exception no heteroagglutination reactions were obtained with fluids and cells of animals belonging to the same taxonomic class.

4. Closely related (same class in most cases or same order in some) species were found to behave alike with respect to the ability or inability of their cells to react to the various fluids, and the fluids of closely related species exhibited similar reactivity.

5. The diluted seminal fluids gave reactions that in most cases paralleled those obtained with the body-fluids.

6. Absorption tests revealed the presence of at least four distinct heteroagglutinins in Patiria body-fluid, and indicated that each is characterized by a broad group-specificity similar to that previously reported for lobster-serum.

7. The general bearing of these results on fertilizin-antifertilizin reactions and on phylogenetic problems is briefly discussed.

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DISTRIBUTION AND PROPERTIES OF INTRACELLULAR ALKALINE PHOSPHATASES [1]

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The chemical and structural composition and the interrelationships of the components of the cell have long been the concern of biologists, in hope of revealing the fundamental processes within the structural and functional unit of all living matter. The early studies of Miescher upon the chemical composition of the nucleus were contemporary with the studies of the structure of the nucleus by van Beneden, Flemming, and others, which later culminated in the firm establishment of the nucleus as the site of phenomena of central importance in the mechanism of Mendelian heredity. The biochemical line of investigation, started by Miescher, has had no such tradition of continuous progress as have the studies of chromosomal structure; but as a result of sporadic advances following the appearance of new methods and techniques, certain facts about the chemical composition of the cell and the nucleus have become firmly established. Miescher noted that the nucleus had a high content of organically bound phosphate; later researches (Levene and Bass, 1931) have fully confirmed this, and have led to a fairly comprehensive knowledge of the chemical composition of nucleic acid, the substance in which all this nuclear phosphate is contained. The unit of nucleic acid is the mononucleotide, a phosphoric acid ester of pentose sugar in glucosidic linkage with one of the purine or pyrimidine heterocyclic bases. The nucleic acids, which have been intensively studied, have been found to be polynucleotides with four different bases, and in their native form they are very highly polymerized, consisting in some cases of hundreds or thousands of nucleotides. There are two types of nucleic acid with respect to the sugar; one contains a pentose which whenever identified has been found to be d-ribose, and the other a desoxypentose found to be d-ribodesose. The wide application of the Feulgen cytochemical test for desoxypentose indicates that nucleic acid with this sugar does not occur outside of the chromatin of the cell nucleus (that is, in the chromosomes), and actual analysis of isolated nuclei and chromosomes has confirmed the view that desoxypentose is the characteristic nucleic acid of chromatin (Mirsky and Pollister, 1942). The name chromonucleic acid has recently been proposed (Pollister and Mirsky, 1943) for desoxypentose nucleic acid as a convenient way of emphasizing this striking limitation in distribution. Nucleic acids can also be located within the cell by means of the intense specific absorption of ultra-violet light by purine and pyrimidine bases, and Caspersson (1936, 1940) has made use of a combination of microspectroscopy and the Feulgen nucleal reaction to determine the distribution of the Feulgen negative pentose nucleic acids. This was found to be largely in the cell cytoplasm, the occurrence of pentose nucleic acid in the nucleus being confined to the plasmosome,

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or the non-chromatin nucleolus. The term plasmonucleic acid has been proposed to embody this information concerning the distribution of pentose nucleic acid. The nucleic acid makes up but a part, usually less than half, of the content of the nucleus. The remainder is apparently largely protein (Mirsky and Pollister, 1942). Much of this is of the basic type, either histone or protamine, but there is also evidence of protein of globulin type (Caspersson, 1936; Mazia and Jaeger, 1939).

In view of the fact that the nuclear chromatin contains a large amount of phosphate in desoxyribose nucleic acid, it is of considerable interest that definite evidence of phosphatase activity within chromatin has been found. Almost simultaneously the author (Krugelis, 1942) and Willmer (1942) reported the demonstration of alkaline phosphatase activity within chromosomes. This discovery stimulated the present study, the purpose of which is two-fold. The first purpose was to investigate the occurrence of alkaline phosphatase in all parts of the cell, and to investigate further the correlation of desoxyribose nucleic acid with alkaline phosphatase activity in the chromosomes. The material which seemed most promising for this first part of the study were the salivary gland chromosomes of Drosophila larvae, because of their peculiar structure which manifests itself in bands or regions rich in desoxyribose nucleic acid, alternating with interband regions poor or lacking in desoxyribose nucleic acid. This part of the investigation can be expressed in the form of a question: Is phosphatase activity located in the chromosomal regions rich in desoxyribose nucleic acid? The second part of the investigation followed from the first, and included the use as substrate of actual chemical substances that are found in the cell, in order to discover whether alkaline phosphatase is capable of hydrolyzing naturally-occurring substances with which its activity seems to be physically associated. If the activity of phosphatase is located in regions rich in desoxyribose nucleic acid, is it capable of hydrolyzing desoxyribose nucleic acid and other naturally occurring substances in the cell? This part of the investigation it was hoped would lead to some suggestion as to the possible role of phosphatase activity within the cell and the chromosome.

Methods and Materials

The histochemical method used was that developed by Gomori (1939, 1941); when using smeared insect material it was found necessary to make some modifications of the preliminary steps. This process involved dissecting, and smearing the insect organs in isotonic saline, or Ringer's, made according to Buck and Melland (1942). After smearing, the material was fixed in 95 per cent ethyl alcohol vapors for one hour, then fixed in liquid 95 per cent ethyl alcohol for two hours. Material fixed as short a time as one hour and as long a time as 48 hours showed little appreciable difference in the results. The coverslips fell off readily in the alcohol, and the smears on the slides were washed briefly in distilled water and then incubated in the substrate solution. The mammalian organs used, namely, the kidney, liver, testis, intestine, and pancreas, were removed after ether anesthesia, fixed in 95 per cent ethyl alcohol for 24 hours, dehydrated in 100 per cent ethyl alcohol, cleared in benzene, and embedded in paraffin in the usual manner. Sections of 5 microns thickness were cut. To avoid any variation in mounting or incubating, sections of the five organs were mounted together on one slide. After deparaffinization, hydration, and washing, sections were ready for immersion and incubation in the substrate solution.

The process from this point on was the same for both the sectioned and the smeared materials. The substrate solution was of the following composition:

2 parts of 0.1 M sodium glycerophosphate (or 0.1 M other organic phosphate ester)
2 parts of 0.1 M calcium nitrate
1 part of 0.1 M veronal buffer at pH 9.4
5 parts of distilled water.

The pH was checked with thymol blue and adjusted to pH 9.0 to 9.2 with NaOH. The incubation was carried out at 25–28° C. and lasted from 12 to 24 hours. Following the incubation in the substrate, the slides containing the smears or the sections were immersed in 0.02 M calcium nitrate for one minute, then into 0.1 M cobaltous nitrate for two minutes, then dilute potassium sulfide for two minutes. After this visualization treatment, slides were washed, dehydrated, cleared, and mounted by the usual histological technique. The results were checked, in some cases, by parallel experiments in which the silver nitrate visualization method (Gomori, 1939) was used. When no activity was obtained in the substrate-incubated slides, the reaction was allowed to proceed for 72 hours to verify the negative results.

At the suggestion of Dr. A. E. Mirsky partially depolymerized nucleic acid was tried as the substrate. This depolymerization was accomplished by using desoxyribonuclease from beef pancreas, made according to the method of McCarty (1946). Desoxyribose nucleic acid is dissolved in distilled water and then placed in a nuclease medium of the following composition:

0.01 per cent magnesium sulfate
0.003 M gelatin
0.025 M veronal-HCl buffer at pH 7.5.

The nuclease is allowed to react upon the desoxyribose nucleic acid for 24 hours in which time the solution becomes clear and loses its viscosity. This change is used as a criterion for the depolymerization of the nucleic acid. This depolymerization leaves the nucleic acid in only a less highly polymerized state; and does not, for example, reduce it to an approximation of tetranucleotide. After the depolymerizing reaction, the solution is heated to 60° C. for fifteen minutes to destroy the activity of the nuclease on the desoxyribose nucleic acid, and then is used as the substrate source of phosphate ester.

The biological materials used in this study were insect and mammalian organs. The salivary glands of the larvae of Drosophila were used for the first part of the experimentation. The larvae were raised at 17–18° C. and at room temperature. The glands were dissected out when the larvae were full grown and had left the food prior to pupation. The species of Drosophila investigated included the Brazilian species *Drosophila pallidipennis* obtained through the generosity of Professor T. Dobzhansky, *D. simulans, D. virilis,* and *D. ananassae* obtained from the stocks at the Biological Laboratories, Cold Spring Harbor, N. Y. The major part of the investigation on Drosophila was done on *Drosophila melanogaster.*

The mammalian organs used were those of mice obtained from the stocks maintained at Columbia University by Professor L. C. Dunn and Dr. S. Gluecksohn-Schoenheimer. The testes were used either after being smeared in the body fluid surrounding the tubules, or after being fixed and embedded as the other organs were.

Yeast nucleic acid and adenylic acid were obtained from the Schwartz Laboratories, New York; thymonucleic (desoxyribose nucleic) acid and nuclease were generously supplied by Dr. A. E. Mirsky of the Rockefeller Institute for Medical Research; guanylic acid, sodium guanylate, and cytidylic acid were obtained from the Levene collection of chemicals at the Rockefeller Institute, also through the kindness of Dr. A. E. Mirsky. Photographic materials were provided from a grant to Columbia University by The Rockefeller Foundation.

Experimental Data

Intracellular occurrence and localization of alkaline phosphatase activity in the salivary gland cells of Drosophila melanogaster

Upon microscopic examination of smears of larval salivary glands of Drosophila, it is evident that in the preparations which have been incubated in the substrate, there is a much greater precipitate of cobaltous sulfide, indicating alkaline phosphatase activity, than in the smears incubated in control solutions. A diffuse precipitate occurs throughout the cytoplasm, but there is a much higher density within the nucleus (Fig. 1). This agrees with the observations of Moog (1944), on the tissues of the chick embryo that the nuclei were never less reactive than the cytoplasm and that the nuclei, unlike the cytoplasm, were never negative in reaction. Dounce (1943) using nuclei isolated from rat liver, also found that alkaline phosphatase activity is much greater in nuclei than in total liver.

Within the nucleus the precipitate is sharply localized in the chromosomes and nucleolus. The nuclei of the control slides show slight, if any, precipitate, with the exception that there is always some cobaltous sulfide in the control nucleoli (Fig. 2), but this is always considerably less in amount than that in nucleoli of substrate-incubated preparations.

The most dense precipitate is found within the chromosomes. When smeared in Ringer's solution, which is necessary to preserve the activity of the enzyme, the details of the chromosome structure are by no means as distinct as in the acetic acid smears that have been used in mapping bands. Nevertheless one can be certain that the cobaltous sulfide within the chromosome is concentrated in transverse bands, alternating with regions of very little or no precipitate (Figs. 3 and 5). This observation was reported earlier (Krugelis, 1945) and it has recently been confirmed by Danielli and Catcheside (1945) who compare the individual bands with specific regions of the cytogenetic chromosomal maps. In the preparations of Figures 3 and 5 the identity of the regions of phosphatase precipitate with the bands of acetic acid smears was established by a direct cytological observation. This method made use of the fact that the cobaltous sulfide precipitate is not altered by the standard Feulgen cytochemical procedure, that it becomes possible to superimpose the nucleal reaction on a phosphatase test. Figure 4 is from such a preparation; and comparison with Figure 5, the same chromosome after the phosphatase reaction but before the Feulgen test, shows that the bands in which the enzyme activity is localized correspond strictly with the Feulgen positive bands that form the basis of the familiar cytogenetic maps. The superposition of the second procedure merely changes the brown band to a purple brown, which is clearly a combination of cobaltous sulfide and basic fuchsin. This combination of the two cytochemical tests shows, in a vivid manner, that the highest level of alkaline phosphatase activity within the salivary gland cell occurs

in exactly the region that contains a very high concentration of organic phosphate in the form of desoxyribose nucleic acid.

Properties and distribution of intracellular alkaline phosphatases

The occurrence of the enzyme activity and the desoxyribose nucleic acid within the same parts of the salivary gland chromosome suggests a functional relationship, which suggestion was put to test by using other phosphate esters as substrates for the enzyme activity. In order to elucidate a more specific picture of possible enzyme activity *in vivo* within the nucleus and the cell, esters were chosen which are normally present in the cell, and which the enzyme would consequently be expected to hydrolyze. The cellular substrates used, along with sodium glycerophosphate as the standard, were purine and pyrimidine nucleotides, ribose nucleic acid (presumably not highly polymerized), and desoxyribose nucleic acid (both polymerized and partially depolymerized). These substrates were tried on various mouse tissues for the presence of the enzyme activity in the cells. The testis was used for the chromosomal localization, since the other tissues contained nuclei which were mainly in the resting stages. In the results the black-brown precipitate of cobaltous sulfide indicates the site of alkaline phosphatase activity on the specific organic substrate in the incubating solution, and the density of the precipitate may be regarded as a measure of the intensity of the enzyme activity.

The photomicrographs (cf. Plates II, III, and IV) illustrate the results of these experiments; and roughly quantitative estimates of the intensities of the reaction are summarized in Table I. With sodium glycerophosphate and the nucleotides as substrates, the precipitate is not sharply localized, though it is always more dense in the nucleus and chromosomes than in the cytoplasm. By contrast, the reactions with depolymerized nucleic acids result in precipitates which are restricted to particular parts of the cell. On the slide in which depolymerized chromonucleic (desoxyribose nucleic) acid has been used as substrate the dark nuclei and chromosomes stand out very distinctly against a colorless cytoplasmic background, an appearance not unlike that of a Feulgen preparation. On the other hand, if plasmonucleic (ribose nucleic) acid is the substrate, the slide as a whole is intensely dark due to cytoplasmic precipitate, and the nuclei appear as light areas. The two types of slides compare with one another somewhat as do a photographic positive and negative. The chromonucleic acid precipitate is not uniformly distributed throughout the

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EXPLANATION OF PLATE I

The dark precipitate indicates the phosphatase activity. Photomicrographs made from smear preparations. Figures 1, 3–5 show phosphatase activity with sodium glycerophosphate as the substrate.

FIGURE 1. Alkaline phosphatase activity in salivary gland of *Drosophila melanogaster* larva. Magnification is 200 ×.

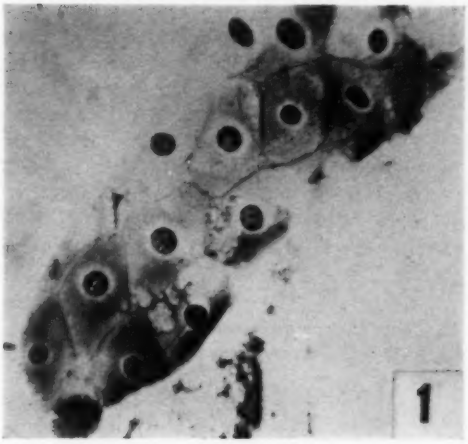
FIGURE 2. Salivary gland nucleus from control slide which had no substrate for the alkaline phosphatase activity. Magnification is 1000 ×.

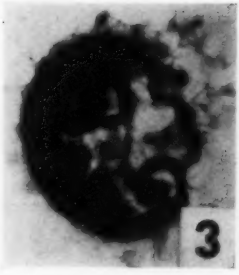
FIGURE 3. Salivary gland nucleus showing alkaline phosphatase activity. Magnification is 1000 ×.

FIGURE 4. Salivary gland chromosomes with the Feulgen reaction applied over the phosphatase reaction precipitate. Magnification is 3000 ×.

FIGURE 5. Salivary gland chromosomes (same chromosomes as Fig. 4) showing alkaline phosphatase activity before Feulgen reaction applied over the precipitate. Magnification is 3000 ×.

PLATE I





nucleus. Instead it occurs only within the formed structures of the nucleus, the chromatin and the plasmosome nucleolus.

In an attempt to find enzyme differences involved in producing such specific localizations of alkaline phosphatase activity, these reactions were subjected to various conditions which might alter the reaction environment. Exposures to temperatures of 55° C. or over for a period of 5 minutes or longer produced complete irreversible inactivation of all the enzyme reactions. Magnesium ions in the concentration of 0.01 M magnesium sulfate showed some activation with the cytoplasmic reaction on ribose nucleic acid, and little or no increase on the nuclear and the general reactions. This very slight activating effect is in agreement with Schmidt and Thannhauser's (1943) observation that there is but slight effect of magnesium on intestinal alkaline phosphatase activity with sodium glycerophosphate as substrate.

TABLE I

Phosphatase reaction with different substrates

Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate	1	2	1	2	0	2	±1	2	3	2
Adenylic acid, guanylic acid, cytidylic acid	1	2	1	2	0	2	0	2	1	2
Desoxyribose nucleic acid	0	0	0	0	0	0	0	0	0	0
Depolymerized desoxyribose nucleic acid	0	2	0	2	0	2	0	2	1	2
Ribose nucleic acid	2	±1	2	1	1	±1	2	±1	2	1

Density of cobaltous sulfide precipitate recorded from visual estimates. 0, no reaction; ± doubtful; 1, definite reaction; 2, strong reaction; 3, very strong reaction. Each record based on microscopic examination of at least 20 slides. Column c, cytoplasmic precipitate; column n, nuclear precipitate.

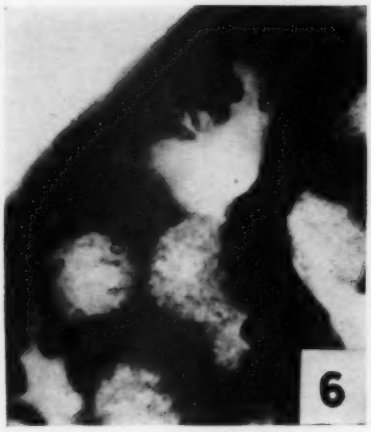
Cyanide ions in the concentration of 0.01 M potassium cyanide completely inhibited all three types of reaction.

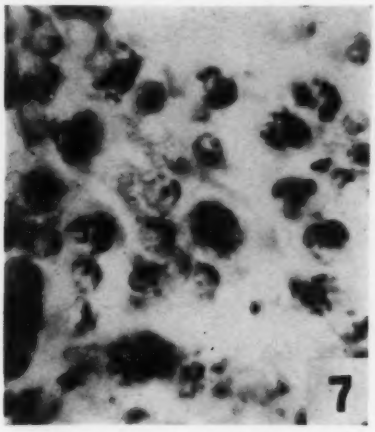
Arsenate ions in the concentration of 0.01 M sodium arsenate showed a more selective effect on the different reactions than did the environmental influences mentioned above. In the presence of arsenate, the alkaline phosphatase activity with the nucleic acids as substrates is completely suppressed. The glycerophosphate splitting, by contrast, in most cases proceeds at its normal rate in a medium which contains arsenate. A roughly quantitative estimation of the inhibition or suppression of activity by arsenate ions is presented in Table II.

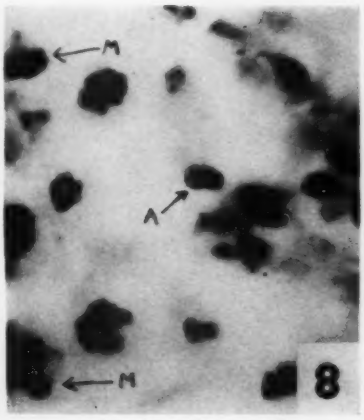
DISCUSSION

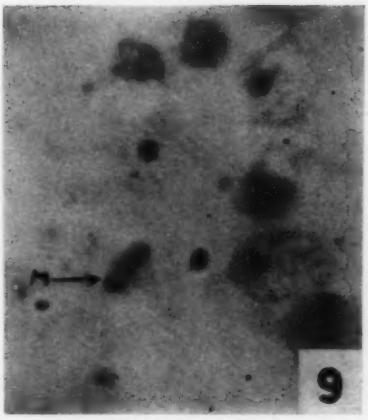
The differences in site of alkaline phosphatase activity in the same tissues when using different phosphate bearing substrates indicate localized reactions of three

PLATE II









Photomicrographs from 5 micra thick sections. Figures 6–8 show alkaline phosphatase activity with sodium glycerophosphate as substrate.

FIGURE 6. Small intestine of mouse showing activity of the enzyme distributed in the mucosa and submucosa layers. Magnification is 1000 ×.

FIGURE 7. Testis of mouse. Magnification is 1000 ×.

FIGURE 8. Testis of mouse. *M* indicates chromosomes in metaphase. *A* indicates chromosomes in anaphase. Magnification is 1000 ×.

FIGURE 9. Testis of mouse from a control slide with no substrate for enzyme activity applied. *M* indicates chromosomes in metaphase. Magnification is 1000 ×.

types, as follows: first, a general alkaline phosphatase reaction in both the cytoplasm and the nucleus, as in the case where sodium glycerophosphate and nucleotides are used as substrates; second, a definite nuclear reaction with little or no activity in the cytoplasm, as in the case of depolymerized desoxyribose nucleic acid as substrate; third, a definite cytoplasmic reaction with little reaction in the nucleus, as in the case of ribose nucleic acid as substrate. Since the enzymes cannot be isolated at this time, and since the nuclear and the cytoplasmic reactions are not absolutely specifically nuclear or cytoplasmic, they will be listed as reaction types with reference only to the location of the activity observed. With the different substrates used, the total reaction of phosphatase activity is somehow produced, and this is detected by the location of the cobaltous sulfide precipitate. The final reactions produced under a

TABLE II

Intensity of precipitate of phosphatase reaction in different tissues using different substrates with and without arsenate ions

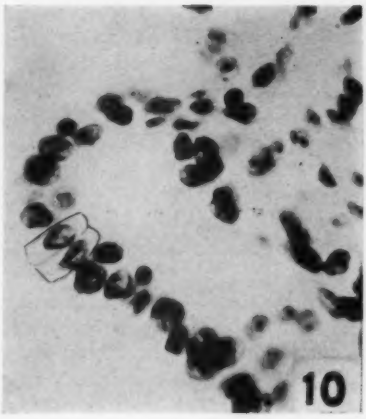
Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate										
No arsenate ions	1	2	1	2	0	2	±1	2	3	2
With arsenate ions	1	1*S1	1	2	0	0*S3	±1	2*S0	2	±1*S1
Depolymerized desoxyribose n. acid										
No arsenate ions	0	2	0	2	0	2	0	2	1	2
With arsenate ions	0	0*S3	0	0*S3	0	0*S3	0	±1*S2	0	0*S3
Ribose nucleic acid										
No arsenate ions	2	±1	2	1	1	±1	2	±1	2	1
With arsenate ions	0	0*S3	2	0*S3	0	0*S3	0	0*S3	2	1*S0

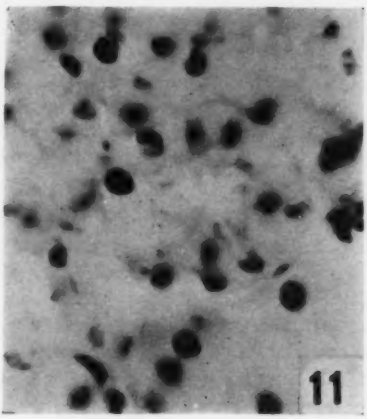
*S = suppression estimates.
S0 is no suppression.
S1 is slight suppression.
S2 is much suppression.
S3 is complete suppression.
Each record represents observation on 8 experimental slides.

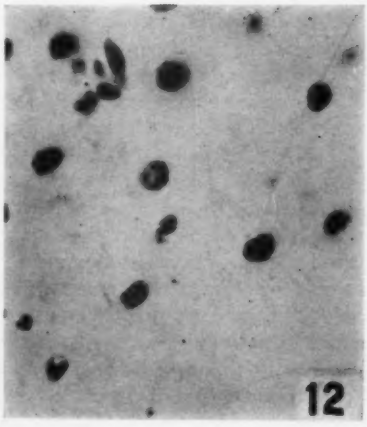
variety of substrate conditions must be due to at least three different enzymes, working in at least two different complexes.

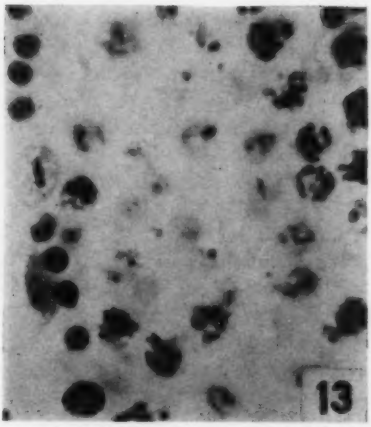
The suggestion that the reactions observed are due to complexes of enzymes is based on the chemical structure of nucleic acids. The ribose and desoxyribose nucleic acids, which were applied as substrates, are polymers of mononucleotides, which are considered to be linked to each other by an ester bond between the phosphate group of one nucleotide and the sugar group of the neighboring nucleotide, thus making the nucleic acids diesters of phosphoric acid all along the chain except in the terminal monoester of phosphoric acid. The total phosphatase action on the nucleic acids might be considered as essentially due to a specific desoxyribose nucleic acid phosphodiesterase, and a specific ribose nucleic acid phosphodiesterase action liberating mononucleotides of the nucleic acids, which then are hydrolyzed by a phospho-

PLATE III









Photomicrographs from 5 micra sections. Figures 10–13 show alkaline phosphatase activity with depolymerized desoxyribose nucleic acid as substrate.

FIGURE 10. Small intestine of mouse showing the distribution of the enzyme activity in the mucosa layer. Magnification is 1000 ×.

FIGURE 11. Kidney of mouse showing enzyme activity in the proximal tubules seen in cross-section. Magnification is 1000 ×.

FIGURE 12. Pancreas of mouse showing distribution of enzyme activity. Magnification is 1000 ×.

FIGURE 13. Testis of mouse showing enzyme activity. Magnification is 1000 ×.

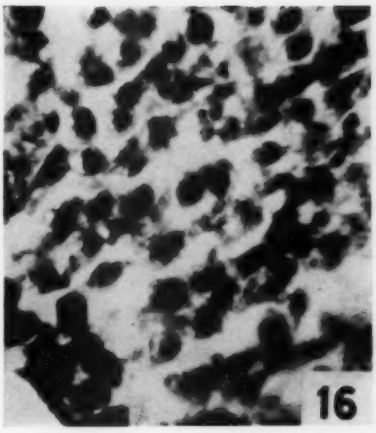
monoesterase to liberate the inorganic phosphate. Thus the precipitate formed when the nucleic acids are applied as substrates is due to the activity of at least two different enzyme complexes, as follows: in the *cytoplasm*, first, a specific phosphodiesterase ("plasmonucleodiesterase") liberates ribonucleotides; second, phosphomonoesterase liberates inorganic phosphates from these nucleotides; in the *nucleus*, first, a specific phosphodiesterase [3] ("chromonucleodiesterase") liberates desoxyribosenucleotides; second, phosphomonesterase liberates inorganic phosphate from these nucleotides. This specificity of the diesterase follows from the fact that ribose nucleic acid will not serve as substrate for the nuclear diesterase activity, nor will desoxyribose nucleic acid serve for the cytoplasmic activity. With regard to the monoesterase activity, however, no such specificity has been detected, for the products of ribose nucleic acid hydrolysis serve equally well as substrates for phosphate production in either nucleus or cytoplasm.

While the action of the phosphodiesterase in freeing mononucleotides from lower polynucleotides (depolymerized nucleic acid) presumably can only occur by attack upon the linkage between the phosphate of one nucleotide and the sugar of the adjacent unit, it is evident that there is considerable restriction upon the exact nature of the bond which can be so attacked. If the enzyme were able to hydrolyze the bond at many points along a nucleic acid chain (consisting, let us say, of 2000 nucleotides) the diesterase should also function as a depolymerase, and a phosphate precipitate should be formed when the long, polynucleotide chains (polymerized nucleic acid) are used as substrate. Even after 72 hours action, however, there is no visible precipitate under these conditions, in contrast to the depolymerized nucleic acid experiments in which a dense precipitate is formed in a few hours. The type of bond which the diesterase can attack is evidently one which is enormously multiplied by a process of depolymerization. A most obvious view of enzyme specificity that would agree well with these facts is that the diesterase can attack only the bond between a terminal nucleotide and the penultimate nucleotide. Considering, for example, an extreme case, depolymerization of a 2000 unit polynucleotide chain to the minimum tetranucleotide should increase the number of bonds which such a specific diesterase can attack by a factor of 500. If specific terminal hydrolysis is the mechanism, the amount of mononucleotide that would become available by diesterase action upon the end of a highly polymerized nucleic acid chain would surely give an amount of phosphate precipitate so slight that it would be cytologically undetectable.

It is highly important to the question of the functional significance of the distribution of the phosphatases acting on the nucleic acids, that this actually coincides in a striking manner with the known locations of desoxyribose and ribose nucleic acids within the cell. The phosphatase activity on depolymerized desoxyribose nucleic acid is restricted to the chromatin of the nucleus, the only part of the cell in which this type of nucleic acid is found. By contrast, the ribose nucleic acid phosphatase activity is in the cytoplasm, a region in which only ribose nucleic acid has ever been demonstrated. (The only marked discrepancy is the occurrence of desoxyribose

[3] Concerning the existence of this nuclear diesterase, there is some possible supporting evidence from the work by Mazia and Ballentine, reported by Mazia (1941), on an intranuclear enzyme from Arbacia eggs. Their enzyme, termed polynucleotidase, was active at a pH 9.0 and was capable of reacting on desoxyribose nucleic acid still in a polymerized form.

PLATE IV



Photomicrographs from 5 micra thick sections. Figures 14–17 show alkaline phosphatase activity with ribose nucleic acid as the substrate.

FIGURE 14. Small intestine of mouse showing the enzyme activity in the cells of the mucosa layer. Magnification is 1000 ×.

FIGURE 15. Small intestine of mouse showing goblet cells in the mucosa layer and the distribution of the enzyme activity. Magnification is 1000 ×.

FIGURE 16. Testis of mouse showing the enzyme activity. A denser precipitate is found in the nuclei of testis cells than in nuclei of other tissues under the conditions of the same substrate. Magnification is 1000 ×.

FIGURE 17. Kidney of mouse, showing enzyme activity in the proximal tubules in cross-section. Magnification is 1000 ×.

nucleic acid phosphatase activity in the nucleolus, which, since it is Feulgen negative, is considered to contain ribose nucleic acid.) [4]

Certain possible *in vivo* functions of these alkaline phosphatases are at once obvious. Not only can the nuclear diesterase split off terminal mononucleotides, as in these experiments, but in the reverse direction, it may conceivably catalyze the terminal growth in the development of nucleotide chains. One may easily picture the later stages of synthesis of a full length nucleic acid chain as involving the cooperation of two enzymes: the diesterase slowly builds up short chains by terminal growth and this is followed by the action of the depolymerase type of enzyme catalyzing the union of these short chains into the long complex which is such an important structural component of a chromosome.

While it is also obvious that catalysis of the synthesis of a mononucleotide from a nucleoside by phosphomonesterase is an essential step in nucleic acid synthesis, one's attention here tends rather to focus on the possibilities of dephosphorylation of nucleotide as a source of energy for nuclear and cytoplasmic reactions. Thus energy for synthesis of chromosomes and their products may or may not be available according to whether the nucleotides are structurally isolated from phosphomonesterase activity by being bound in nucleic acid chains, or whether as a result of a successive action of nuclear depolymerase, and "chromonucleodiesterase," there is mononucleotide available for dephosphorylation. Similarly the actual availability of energy for such cyclic nuclear mechanical processes as chromosome coiling and mitotic movement may be dependent upon a cycle of binding and release of mononucleotide from its nucleic acid storehouse.

Summary

1. Using the histochemical test for alkaline phosphatase reaction in the larval salivary glands of several species of Drosophila, activity was found to be present in three main parts of the cell; the cytoplasm, the nucleolus, and the chromosomes.

2. Phosphatase activity was found rather generally distributed in both the cytoplasm and the nucleus. Within the larval salivary gland chromosomes, the enzyme activity was localized in those chromosomal regions which are Feulgen positive, and thus corresponds to the regions containing large concentrations of desoxyribose nucleic acid.

3. Different naturally occurring phosphate bearing substances were used as phosphatase substrates on mouse tissues, and resulted in demonstration of three different types of phosphatase reactions based on the localization of the enzyme activity.

a) A general reaction with phosphatase activity located in both nucleus (nucleolus and chromosomes) and the cytoplasm was present when sodium glycerophosphate and nucleotides were used as substrates.

b) No phosphatase reaction occurred on polymerized desoxyribose nucleic acid, but a specific nuclear reaction (nucleolus and chromosomes) was present when nuclease-depolymerized desoxyribose nucleic acid was used as a substrate.

[4] We do not actually know the location of the possible substrates for the diesterase activity, since the methods for localizing nucleic acids do not, in all likelihood, preserve any but the high polymers, and it may well be that the lower polymers of the sort used as substrates are not the same in distribution as the larger complexes. In such a difference may lie the explanation of the discrepancies above.

c) A strong cytoplasmic reaction with slight reaction in the nucleus was present when ribose nucleic acid was used as a substrate.

4. Subjection to several environmental variables produced little further evidence as to the differences among these three types of localized reactions.

5. The three types of alkaline phosphatase reactions observed were suggested to be due to at least two phosphodiesterases and a phosphomonesterase.

6. The nuclear phosphatase complex and the cytoplasmic phosphatase complex each probably consist of a specific phosphodiesterase, which splits the ester linkage between the phosphate of one nucleotide and the sugar of the neighboring nucleotide, and a phosphomonesterase which splits the second ester linkage and liberates inorganic phosphate.

Acknowledgment

I wish to express my appreciation and gratitude to Professor H. Burr Steinbach for originally bringing to my attention the cytochemical possibilities of the alkaline phosphatase test; to Professor Arthur W. Pollister for his advice and encouragement throughout the course of the work and for his help in the preparation of the manuscript; to Dr. Alfred E. Mirsky for advice and essential materials; and to Dr. Robert Ballentine and Dr. Jack Schultz for many helpful suggestions.

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PHYSIOLOGY OF INSECT DIAPAUSE: THE ROLE OF THE BRAIN IN THE PRODUCTION AND TERMINATION OF PUPAL DORMANCY IN THE GIANT SILKWORM, PLATYSAMIA CECROPIA

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The phenomenon of insect diapause presents an exceptionally clear statement of one of the most important problems in biology; to wit, the nature of the factors that preside over cellular growth and differentiation. For with the onset of diapause and through the workings of internal physiological mechanisms still to be elucidated, growth suddenly comes to a standstill and the animal for months thereafter persists in a genuine state of suspended development. With the termination of diapause the rapid tempo of cellular activity returns and metamorphosis continues where it had left off. Thus whatever may be the inner mechanism for the induction and termination of diapause, it must have the capacity to turn morphogenesis off and on in a most striking way.

The study of this phenomenon has not failed to claim the attention of a large array of investigators. For example, even in 1932, Cousin was able to cite 347 papers in a review of the literature. That this extensive literature has so imperfectly advanced our knowledge of diapause is apparently due to the fact that most investigations have been carried out either on muscoid flies, which have a most imperfect and complex diapause, or on the eggs of silkworms and grasshoppers, which are too small to permit extensive manipulations of the individual animal. In the present investigation these difficulties were minimized by working on species of insects that possess a wholly characteristic pupal diapause, and, by virtue of weighing up to 8 grams per individual, are among the very largest insects in America.

Materials and Methods

Pupae of the giant silkworm, *Platysamia cecropia*, were used for the most part, approximately 1200 pupae being studied in a total of 690 experiments. These insects were reared from eggs obtained from fertile females; a lesser number of pupae were secured from dealers. In my experience, this species has never failed to enter into diapause immediately after pupation, thus giving only one brood a year. If the pupae are maintained constantly at room temperature, diapause persists for not less than five months; if they are placed immediately after pupation at a temperature of 3° to 5° C. and chilled for 1½ months or longer, adult moths emerge about 1 to 1½ months after being returned to room temperature. For this reason the stock of material was divided at the outset into two batches, one being placed and stored at 3° to 5° C. until needed ("chilled pupae"), and the other being maintained at room temperature where, as previously described, diapause persists for at least five months ("diapausing pupae").

In a number of experiments related species of saturniid pupae were used; namely, *Samia walkeri*, *Callosamia promethea*, and *Telea polyphemus*.

The most important factor facilitating the investigation was the discovery of a method of continuous anesthesia for insects during operative procedures. This method, utilizing carbon dioxide and described by Williams (1946), permitted extensive and prolonged surgical manipulations without any loss of blood or apparent damage to the pupae. Other procedures will be described as encountered in the following discussion.

Parabiotic Experiments

We have noted that diapausing pupae, after a period of exposure to low temperature, are rendered competent to develop when returned to room temperature, whereas, in contrast, pupae not subjected to chilling remain in diapause for at least five months. With these two types of animals at hand one is therefore in a position to test the fundamental nature of diapause by simply grafting one to the other so that they share the same blood. If diapause results from the *presence* of some factor inhibiting development, then such parabiotic combinations should fail to develop by virtue of the diapausing pupa distributing this inhibitory factor to the chilled individual. To the contrary, if diapause results from the *absence* of some necessary growth factor, both animals should develop, provided the chilled individual can supply double the minimal amount needed by a single animal.

In making these preparations a disc of pupal cuticle plus underlying hypodermis was cut from each pupa and the two animals placed together and held thus by the application of melted paraffin around the site. Most of the pupae were joined at the thoracic tergum (Figs. 1 and 3), but occasionally the junction was accomplished at the head or at the tip of the abdomen. Provided that the underlying heart is not injured and that no bubbles of air are trapped in either animal, such combinations are easily established and a high percentage survive.

In order to demonstrate that the operation in itself is without effects on dormancy, a series of ten diapausing pupae were successfully grafted to diapausing partners. Diapause persisted in each of these animals, adult moths being produced only after a minimum of 5½ months, the usual minimum length of time necessary for the spontaneous termination of diapause at 25° C. To the contrary, when diapausing pupae were joined to previously chilled individuals, the diapause in all viable preparations was terminated. In a series of 15 such combinations the pairs emerged as fully formed, active moths in an average of 41 days. Metamorphosis was complete both externally and internally, the only defect being a failure of the wings to expand after emergence (Fig. 2). This activation was not species- or, indeed, genus-specific, for it was possible to terminate the diapause of *Platysamia cecropia* by joining them to previously chilled pupae of *Telea polyphemus* (Figs. 3 and 4). Furthermore, sexual differences were without significance, for male pupae had the capacity to induce development of females, and vice versa.

A striking feature of all these parabiotic combinations is the fact that the animals invariably grow together so as to be connected by a pedicle, a phenomenon first noted by Crampton (1899) and later by Wigglesworth (1936) and Bodenstein (1938) in grafting procedures on insects. We shall have occasion subsequently to consider this union more fully, but in the present analysis suffice it to say that the

epithelial pedicle becomes externally chitinized and by way of its lumen permits a circulation of blood between the two animals.

In the earlier preparations the blood of the diapausing and chilled pupae in parabiosis was daily propelled to and fro by pressing accordion-like on the abdomen of each pupa alternately. This was later found to be wholly unnecessary, since development begins just as promptly without such forced mixing. It may also be noted that the completion of adult formation in the previously chilled animal occurs about 1½ days earlier than in the diapausing partner (Fig. 5). This results from a corresponding delay in the initiation of development of the diapausing pupa. At all stages in adult differentiation the chilled pupa is therefore approximately 1½ days in advance of the diapausing partner.

Thus these initial parabiotic experiments indicate some of the essential features of diapause. In general, they support the proposition that diapause results from the absence of a non-species-specific growth factor that is able to pass in parabiotic preparations from the activated to the dormant individual and evoke the initiation of adult development in the latter also.

Brain Implantation into Diapausing Pupae

If the termination of diapause is, indeed, accomplished by the action within the previously chilled pupa of a factor necessary for adult development, then it should be possible to demonstrate the organ in which this factor arises. For this purpose, various tissues and organs were removed from chilled pupae and implanted singly into diapausing individuals. When experiments of this sort were carried out, it was found that only one organ in the chilled pupa has the power to evoke development of diapausing pupae and that this organ is the brain itself. When the brain is removed from a chilled pupa and implanted into the head, thorax, or abdomen of a diapausing pupa, the latter is invariably induced to undergo adult development. Furthermore, *Platysamia cecropia* can be activated by the brains of *Samia walkeri, Callosamia promethea,* or *Telea polyphemus*—and, in fact, as far as the termination of diapause is concerned, there is a lack of species- and genus-specificity of brains among all these Lepidoptera tested. No other organ in the chilled pupa apparently possesses this power.

This effect of chilled brains is in marked contrast to that of diapausing brains,

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Explanation of Plate I

Approximately Life Size

Figure 1. Brainless, diapausing pupa of *P. cecropia* grafted to a chilled pupa of the same species.

Figure 2. Animals in Figure 1, after adult formation. The two insects have grown together and developed essentially simultaneously.

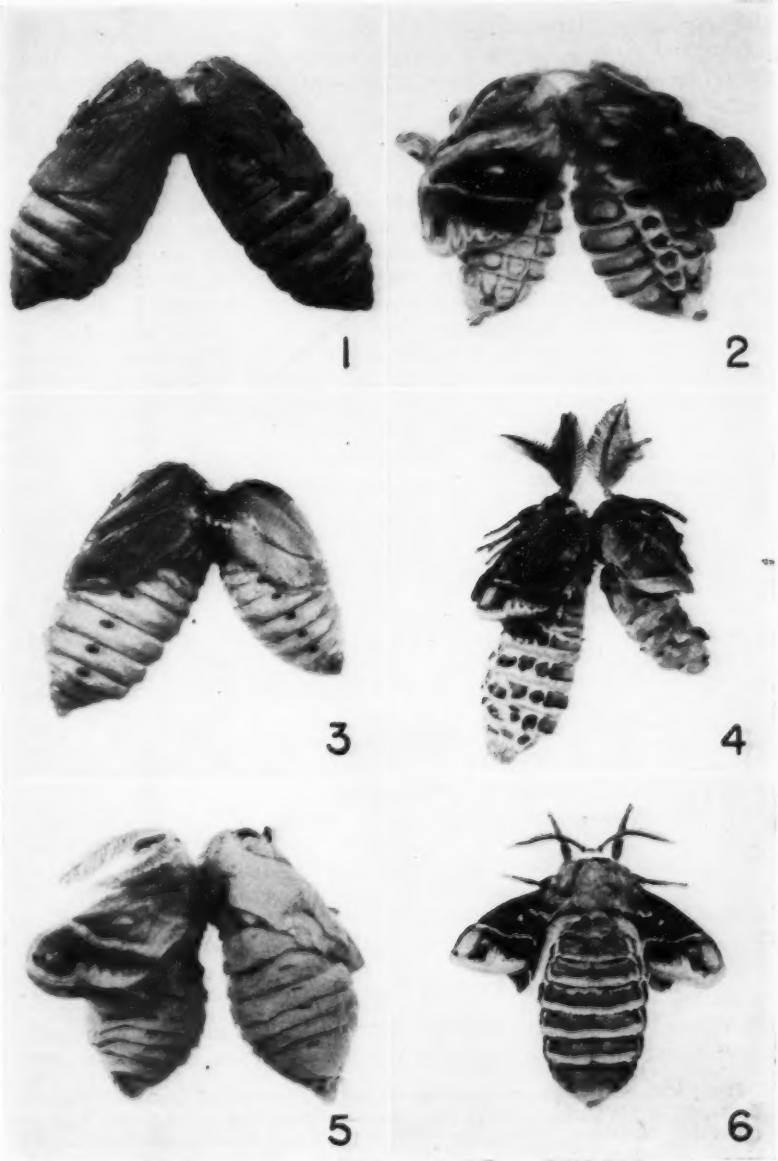
Figure 3. Brainless, diapausing pupa of *T. polyphemus* grafted to a chilled pupa of *P. cecropia.*

Figure 4. Animals in Figure 3, after adult development.

Figure 5. Parabiosis between two pupae of *P. cecropia,* removed from pupal cuticle before the completion of adult development. Development of the chilled pupa is 1½ days in advance of that of the brainless, diapausing pupa.

Figure 6. Adult Cecropia moth produced by a brainless, dipausing Cecropia pupa, whose diapause was terminated by implantation of a brain from a chilled Polyphemus pupa.

PLATE I



for the latter fail to terminate dormancy even when as many as eight are implanted into a single pupa.

The lack of species-specificity of implanted, chilled brains suggested the possibility that the effect might, conceivably, be mediated by an activation of the endogenous brain of the diapausing, "host" pupa itself. For this reason the vast majority of subsequent experiments were carried out on diapausing pupae from which the brains had been removed.

Removal of Brain

The operation devised for this purpose is easily performed, as follows. The insect, anesthetized with carbon dioxide, is placed on its back and by means of a sharp scalpel a rectangular window of pupal cuticle excised from its face. The underlying, semi-transparent hypodermis is thus exposed. With microscissors the latter is trimmed away, along with the tracheae traversing the operative field. In this procedure the frontal ganglion is frequently removed, but this has been found to be inconsequential. The brain now lies exposed and can be floated up toward the operator by pressing the pupal abdomen so that further dissection is performed in the pupal blood. With finely ground jeweler's forceps the nerves passing laterally to the site of the future adult eyes are grasped and broken. In similar fashion each brain hemisphere is carefully broken loose, in turn, from its tracheal supply, from the nerve passing posteriorly to the corpus allatum and corpus cardiacum and from the circumesophageal connective. The brain can then be lifted free and examined in insect Ringer's solution.[1] Taking care to exclude all bubbles, the defect in the pupal chitin is then capped over with a small rectangle of thin, transparent plastic (cut from a plastic cover slip), which is sealed in place with melted paraffin.

The operation was originally performed with due regard to surgical asepsis; this was later found to be of little importance since the pupae are apparently not affected by the usual contaminating organisms. The mortality from the procedure is low and one ends up with a brainless pupa possessing a transparent window at its anterior end.

The defect in the hypodermis is rapidly repaired by a deposit of blood cells, followed by an ingrowth of cells and of tracheoles along the under surface of the plastic slip. Simultaneously, an intervening, delicate, transparent, "chitinous" lamella is elaborated. These relations permit a detailed study of the behavior of the hypodermis beneath the window, and, by means of an Ultropaque, cellular activity has been followed under the oil immersion objective. It may be noted that this local process of repair occurs just as promptly in diapausing as in previously chilled pupae. Notwithstanding this fact, the process of repair is without overall effects on dormancy.

Brain Implantation into Brainless Diapausing Pupae

The removal of the brain from diapausing pupae prior to using the animals experimentally proved to be an exceptionally significant maneuver. For whereas, as

[1] This physiological solution was originally devised by Ephrussi and Beadle (1936) for studies of Drosophila, but it works equally well for the Lepidoptera used in the present experiments. I am indebted to Dietrich Bodenstein for calling my attention to its composition, which is as follows: NaCl, 7.5 gm.; KCl, 0.35 gm.; and $CaCl_2$, 0.21 gm., per liter of water.

we have previously noted, diapausing pupae kept at 25° C. begin to escape spontaneously from diapause after about five months, no such activation occurs if the brain is removed. Among approximately 400 such pupae there has not been a single case of spontaneous development. It is therefore apparent that by removing the brain the pupa is maintained in permanent diapause. Such pupae remain alive for up to a year and finally die of dessication. Yet at any time during this period the diapause can be terminated by implanting into the brainless pupa the brain of a previously chilled animal (Fig. 6). Data in regard to a series of such pupae are given in Table I.

TABLE I

Evocation of Adult Development of Brainless Diapausing Pupae by Implantation of Brains from Chilled Pupae

Species of host	Species of implanted brain	Number of experiments	Aver. time for adult emergence
P. cecropia	*P. cecropia*	16	35 days
P. cecropia	*T. polyphemus*	2	89
P. cecropia	*S. walkeri*	3	72
P. cecropia	*C. promethea*	2	63
T. polyphemus	*T. polyphemus*	2	64
T. polyphemus	*S. walkeri*	2	28

Manifestly, these experiments demonstrate that the termination of diapause requires the presence of an activated brain, in the absence of which adult development of these insects has not been observed. The conclusion is also self-evident that the termination of dormancy after diapausing pupae have been chilled results from the action of low temperature in rendering the brain able to evoke adult development. The other tissues in the diapausing pupa do not require such exposure to cold, for they are rapidly activated by implanting a brain which, alone, has been chilled.

This fact can also be readily demonstrated by removing strips of integument from diapausing pupae and implanting them into previously chilled pupae. Such diapausing tissues develop simultaneously with the host: the pupal cuticle is delaminated and a normally chitinized, adult cuticle, complete with scales and hairs, is found in the implant, in exactly the same fashion as described by Piepho (1938a and b) and Kühn and Piepho (1940) in studies of other aspects of insect metamorphosis. Thus the effect of low temperatures in facilitating escape from diapause can be explained solely on the basis of its effect on the brain.

Parabiotic Experiments on Brainless Diapausing Pupae

As soon as the brain was definitely shown to be the source of the factor terminating diapause, ten more parabiotic combinations were prepared, but this time uniting *brainless* diapausing pupae with chilled individuals. Identical results were obtained: the two pupae in each combination grew together by a chitinized, epithelial, blood-filled pedicle and after an average of 44 days emerged as normal, active, adult moths.

Brain Removal from Previously Chilled Pupae

Further information concerning the action of the brain in terminating diapause can be gained from a consideration of the behavior of chilled pupae. We have previ-

ously noted that these animals undergo no apparent development as long as they are maintained at the low temperature, but that within 1 to 1½ months after return to 25° C. the adult moth has fully formed and emerges. It is therefore worthy of note that if the brain of such chilled pupae is removed as soon as the insect is returned to the warm temperature, adult development never occurs and, in the same fashion as described for brainless diapausing pupae, dormancy persists indefinitely until the animal finally dies of dessication. Yet development can at any time be evoked by merely implanting into the head, thorax, or abdomen a brain obtained from another chilled pupa.

This phenomenon was studied more fully as follows. A series of thirty previously chilled pupae was placed at 25° C. and every few days the brains from several of these insects were removed and implanted into brainless diapausing pupae. The results may be summarized most briefly by saying that when the brain is removed within approximately the first 11 days, the brainless donors never show any development; such brains, in turn, evoke the development of brainless, diapausing pupae. In contrast, if the brain is removed from previously chilled pupae after approximately 17 days at 25° C., development continues to produce normal, brainless adults and the removed brains are without effect in terminating the dormancy of brainless, diapausing pupae.

These experiments have been repeated on a large scale, with special attention to the effect of brain removal during the critical period of 11 to 17 days. These more detailed studies were facilitated by establishing, at the outset, a transparent, plastic, facial window in each chilled pupa so that the behavior of the underlying hypodermis could be observed. It was at once apparent that the critical period, 11 to 17 days, was, in a sense, a statistical artifact, since, during this period, each individual achieves threshold activation during an extremely short interval, not exceeding a few hours. The actual critical period for each pupa is signaled by the initiation of hypodermal retraction from the overlying, facial chitin. Prior to this point, removal of the brain prevents development, and such brains evoke the development of brainless, diapausing pupae after an additional latent period of approximately three weeks. The moment hypodermal retraction is initiated, the brain can be dispensed with and such brains, when tested, are inactive.

Thus it is apparent that diapause persists even in chilled pupae until the latter have been exposed to a developmental temperature for an average of two weeks. Consequently, the activation of the pupal brain during exposure to low temperature must be conceived in terms of some physical or chemical alteration in the brain substance whereby the latter is rendered competent to produce or release its stimulating factor during subsequent exposure to a developmental temperature The brain's action is then exerted and, thereafter, metamorphosis can proceed independent of its further participation.

Role of the Corpora Allata

All the evidence so far considered reveals the brain as the organ of paramount importance in engendering and terminating diapause. Thus diapause in these species appears to result from the absence of a factor necessary for adult development, rather than from the presence of an inhibitory factor. The possibility remained, however, that the failure of the brain to exert its effect and the consequent onset

of diapause might, in turn, be due to inhibition arising elsewhere in the organism. The corpora allata were deemed the most likely source of such hypothetical inhibition and for this reason their significance in the production of diapause was studied.

As originally demonstrated by Bounhiol (1938) and subsequently confirmed by Piepho (1940; 1941), the corpora allata of Lepidoptera specifically inhibit pupation during the larval instars and thus oppose the activation of the presumptive imaginal tissues. This finding seemed so significant that comparable experiments were carried out on the caterpillars of *Platysamia cecropia* and *Telea polyphemus*. Although the removal of the corpora allata from caterpillars is a difficult procedure, it was accomplished in a sufficient number of immature (fourth instar) larvae to demonstrate that precocious pupation, indeed, results therefrom, the usual fifth instar being omitted. Furthermore, there is convincing evidence that the function of the corpora allata in inhibiting the imaginal discs disappears during the final larval instar and pupation then ensues. These findings have been considered in some detail for, although they concern pupation rather than adult differentiation, it is easy to see the importance of demonstrating whether, in potentially diapausing insects, the corpora allata once again inhibit the imaginal tissues, or the brain, and thus participate in the induction of diapause.

A series of experiments was therefore performed in which the corpora allata were removed (by a frontal approach) from diapausing pupae. Such pupae [2] invariably continued to diapause normally, and the removed corpora allata when implanted into previously chilled pupae were without effect in inhibiting adult development. As many as six corpora allata have been implanted into a single chilled pupa without retarding metamorphosis.

Similar negative results were obtained in regard to all other organs studied as a possible source of some inhibitory factor. For example, the diapausing brain itself is without inhibitory properties, for as many as six such brains have been implanted into a single chilled pupa without producing diapause. This is also true for the subesophageal ganglion, thoracic ganglion, gonads, imaginal discs, and strips of integument.

Although it cannot be denied that inhibitory factors may play a role in the production of diapause, the sum total of available evidence offers nothing to support this proposition. The brain itself remains the key to the production and termination of diapause in the species studied.

Discussion

The role of the brain in terminating diapause, demonstrated for the first time in the present investigation, can to advantage be compared with its other functions in insect metamorphosis. Thus, in the bug, Rhodnius, the brain is necessary for moulting (Wigglesworth, 1940) and in the Lepidoptera it is now well established that the brain is also required for pupation (Kopec, 1922; Caspari and Plagge, 1935;

[2] It may be noted that the allatadectomized pupae ultimately escaped from diapause after the usual minimum period of 5½ months at 25° C. The resulting moths were wholly normal in all respects and could be induced to mate and lay eggs, which, in turn, were fertile. These findings apparently deny a participation of the corpora allata in the egg production of these species, a function described for them in certain other Orders of insects (Wigglesworth, 1936; Pfeiffer, 1939).

Kühn and Piepho, 1936; Bounhiol, 1938; Piepho, 1938a; Plagge, 1938). A surprising fact is that a role of the brain in imaginal differentiation has been specifically denied by all of these investigators of lepidopteran metamorphosis. The important point is that this conclusion was, without exception, based on studies of continuous, non-diapausing development. For such insects there can be little doubt that adult formation ensues even though the brain is removed from mature caterpillars in the last instar (i.e., after the "critical period" for pupation). The existence of this striking difference between continuous and diapausing development has been pointed out previously (Williams, 1942).

In the present investigation we have seen that all the evidence supports the theory that diapause results from an interruption in the normal processes of adult development. This point of view suggests that the brain is also necessary for evoking adult development in non-diapausing pupae. In non-diapausing individuals the brain may be viewed as having achieved its full developmental function precociously prior to pupation, whereas, in potentially diapausing animals, the brain first controls pupation and then months later after pupation it controls adult formation.

The action of the brain in terminating diapause in these saturniid pupae poses an additional problem of even greater interest; namely, the nature of the factor arising in the brain which so spectacularly evokes in dormant tissues a veritable flood of cellular activity. This problem will be considered in a subsequent communication.

Summary

1. The physiological control of pupal diapause has been studied on a total of approximately 1200 pupae of the giant silkworms, *Platysamia cecropia, Telea polyphemus, Samia walkeri,* and *Callosamia promethea.*

2. The dormancy of diapausing pupae can be terminated readily by grafting them to activated (previously chilled) pupae. The two animals in each parabiotic combination grow together and some factor necessary for adult development passes from the activated to the dormant animal so that both develop simultaneously. This factor is not species- or genus-specific.

3. By implantation experiments the source of the factor terminating diapause is shown to be the brain and in this function a lack of species- and genus-specificity of brains is demonstrated.

4. In these species the well-known action of low temperatures in facilitating escape from diapause results from the effect of cold in rendering the brain competent to terminate domancy. Actual termination of dormancy is accomplished only after the previously chilled brain has been exposed to a developmental temperature for an average of two weeks. The earliest indications of adult development then become evident and the brain, thereafter, is no longer required for the completion of metamorphosis.

5. Therefore, the effect of low temperatures on the brain must consist in some physical or chemical alteration in its substance whereby the latter is rendered competent to produce or release an imaginal-differentiation factor after return to a developmental temperature.

6. No evidence was found to support the theory that diapause results from the

presence of inhibitory factors. In this regard, the functions of the corpora allata are considered in some detail.

7. It is concluded that diapause in these species results from an interruption in the normal processes of development by virtue of a failure of the brain to supply a non-species-specific factor necessary for adult differentiation. Diapause is terminated when this factor is provided.

8. The significance of the brain in the development of diapausing pupae is considered in relation to its other functions, as reported in the literature. Notwithstanding a certain amount of evidence to the contrary, it is probable that even in the absence of diapause the brain plays a vital role in adult formation.

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FEEDING OF OYSTERS IN RELATION TO TIDAL STAGES AND TO PERIODS OF LIGHT AND DARKNESS

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Introduction

The beds of the American oyster, *Ostrea virginica*, are usually situated in inshore areas where tidal currents are strong and their regularity is sharply defined. Because of an almost continuous flow of water generated by the tidal movements over the beds populated with oysters, these mollusks live under continuously changing conditions. There is no doubt that the environmental changes caused by the tides may in certain instances noticeably affect the behavior of the oysters. It has never been satisfactorily demonstrated, however, that some of the vital processes of oysters, such as feeding, are carried on more vigorously during certain stages of the tide.

A review of the literature on this subject shows that the only attempt to study the relation between the feeding activities of oysters and the stages of the tide was made by Nelson (1921) who came to the conclusion that "The times of complete cessation or of commencement of feeding shows a rather definite correlation with the stage of the tide." In his other article on the feeding habits of oysters Nelson (1923) states that in the case of these mollusks relatively little food is taken on the ebb tide. Even as late as 1938 Nelson still refers to his old observations emphasizing the relative inactivity of the oyster during the outgoing tide (Nelson, 1938). These conclusions were accepted without verification by some other investigators and were finally incorporated in the article on oysters in the Encyclopaedia Britannica where it is stated that "The American oyster does not feed late at night and in early morning, and relatively little on the ebb tide" (Orton, 1929). At present, therefore, such behavior of oysters is recognized as an established fact.

In our investigations of various aspects of the biology of oysters of Long Island Sound and its tributaries, especially of Milford Harbor, it was noticed that these mollusks fed actively on the ebb tide and also during darkness. The stomachs of the oysters removed from the water during such periods contained large quantities of food. It was also observed on numerous occasions in the day time that during the last stages of the ebb the oysters populating the shallow flats were wide open and apparently feeding because they were expelling true faeces. These observations suggested that the conclusions expressed by Nelson and Orton probably did not apply to the oysters of the Long Island Sound and Milford Harbor region. However, since our observations were only of an occasional nature, they could not be regarded as entirely reliable. Therefore, to ascertain the feeding habits of our oysters in relation to the tidal stages, and to the periods of light and darkness, a systematic series of experiments was conducted in the Summer and Autumn of 1945. The solution of the problem undertaken was approached by using several methods, each of which contributed toward the correct evaluation of the results of the others.

The authors wish to express their sincere appreciation and thanks to Miss Frances Tommers for her helpful participation in all the phases of this work and especially for the difficult analysis of numerous kymograph records obtained in the course of these studies.

Observations and Experiments

Stomach content

The first method consisted in examining the volume of the stomach content of the oysters collected during different stages of the incoming and outgoing tides. As a rule, the oysters examined were approximately 4 inches in length. The examination was made immediately after the specimens were taken out of the water. After opening an oyster an incision penetrating to the stomach was made through the body wall and the content of the stomach was collected by means of a fine pipette inserted through the incision.

At first it was intended to employ a volumetric method for determining the quantities of food found in the stomachs but, eventually, this method was found unsatisfactory because the bodies of the oysters, regardless of quite a uniformity of their shells, displayed considerable difference in their volume and size. Since small-bodied oysters possessed smaller stomachs and were probably incapable of ingesting quantities of food equal to those of larger individuals, serious errors could be introduced in the interpretation of the results. Therefore, another method, in which each oyster was considered individually, was decided upon. This method consisted in a direct evaluation of the relative quantity of food found in the oyster's stomach. Three classes, namely: "large," "small," and "absent," were established to designate the results of the examination. The oysters which were classified as containing large quantities of food could easily be recognized because, as soon as the incision was made in their bodies, the stomach content freely flowed out. This phenomenon is probably familiar to all investigators who worked with oysters. The animals placed in the second class were those whose stomachs contained only small quantities of food. The third class was composed of oysters with empty stomachs. The animals of the latter group were especially carefully examined to be certain that they contained no food.

The largest number of oysters examined in the course of these studies was collected from the cultivated oyster beds of Long Island Sound. This group, consisting of 1000 individuals, was composed of ten samples, each usually containing 100 oysters. These samples were collected at approximately weekly intervals during June, July, and August 1945. Each of the large samples consisted in turn of ten smaller ones composed of 8 to 12 individuals. The latter samples were dredged from our ten collecting stations established in the Sound. All these stations, located at a depth ranging from 10 to 30 feet at the mean low water stage, were subjected to strong tidal currents. The exact time of the collection of each sample was recorded and later correlated with the stage of the tide.

The collection of samples from all the stations extending along the shore for a distance of about 25 miles usually required from six to eight hours. Thus, it was sometimes possible to continue to collect samples during the entire period of the outgoing or incoming tide. The stations were not always attended in the same sequence to guarantee that the sampling was of a random nature.

The results of the examination of 1000 oysters offered rather conclusive evidence that the mollusks were feeding as actively on the ebb tide as they did during the flood (Table I). As a matter of fact, the per cent of oysters containing a large quantity of food was somewhat higher on the ebb than during the flood. Furthermore, only 6 per cent of the oysters collected during the ebbing tide possessed empty stomachs, while among the individuals dredged during the flood 10 per cent showed a complete absence of food. It is significant that among the oysters collected during the low water stage the per cent of the individuals containing a large quantity of food was higher, and that of the animals with empty stomachs lower than at many other stages

TABLE I

Relative quantities of food in stomachs of 1000 oysters collected at different tidal stages in Long Island Sound, June, July, and August 1945

Stage of tide	Oysters examined	Quantity of food			Per cent		
		Large	Small	Absent	Large	Small	Absent
Flood							
1st hour	41	31	6	4	75	15	10
2nd hour	60	51	2	7	85	3	12
3rd hour	85	58	9	18	68	11	21
4th hour	75	66	5	4	88	7	5
5th hour	85	72	8	5	85	9	6
High water	110	91	9	10	83	8	9
Total	456	369	39	48	81	9	10
Ebb							
1st hour	80	66	11	3	82	14	4
2nd hour	80	69	7	4	86	9	5
3rd hour	120	102	10	8	85	8	7
4th hour	90	76	10	4	85	11	4
5th hour	107	84	13	10	79	12	9
Low water	67	59	6	2	88	9	3
Total	544	456	57	31	84	10	6
Grand total	1000	825	96	79	82	10	8

of the tide. While this observation cannot be interpreted as definite proof that oysters feed most efficiently just prior and during the low water stage, it shows, nevertheless, that they do not cease, or noticeably decrease, their feeding activities during late ebb.

Additional observations on the relative quantities of food in the stomachs of the oysters at the end of the flood and during the entire period of ebb were made in Long Island Sound on August 21, 1945. On that day a station was chosen in 20 feet of water on one of the beds planted with 4-year-old mollusks. The location of the station was designated by a special buoy. The samples were collected at hourly intervals beginning one hour prior to the high water stage (Table II). Altogether eight samples, each composed of 20 oysters, were collected and examined.

The salinity of the water ranged from 27.31 p.p.t. soon after high water to 25.45 p.p.t. at low water.

The results of the examination again showed that in the majority of the oysters the stomachs contained large quantities of food during all stages of the ebb (Table II). The observations also indicated that there was no decrease in the number of oysters with food-filled stomachs parallel with the falling of the tide. On the contrary, the largest number of oysters containing large quantities of food was found only one hour prior to low water.

It is significant that among the oysters examined during the last three hours of the ebb not a single individual was found with an empty stomach. While the presence of food in the oysters dredged during the early stages of the outgoing tide could be possibly explained by assuming that this food was ingested during the last

TABLE II

Relative quantities of food in stomachs of 160 oysters collected at hourly intervals during last hour of flood and during ebb from a station established in 20 feet of water in Long Island Sound, August 21, 1945. Each sample composed of 20 oysters.

Stage of tide	Temperature °C.	Quantity of food		
		Large	Small	Absent
Flood				
5th hour	21.2	11	8	1
High water	20.9	17	3	0
Ebb				
1st hour	21.1	14	6	0
2nd hour	21.0	19	1	0
3rd hour	20.9	17	2	1
4th hour	21.1	19	1	0
5th hour	20.6	20	0	0
Low water	20.4	16	4	0
Total		133	25	2

hour of flood, such an explanation cannot be offered for the presence of food in the oysters examined from three to six hours after the high water stage. Our observations performed under laboratory conditions on oysters kept in water of 20.0° C. showed that these mollusks pass the particles of food through their entire digestive system from 1 hour 20 minutes to approximately 2 hours and 30 minutes. Therefore, it seems rather improbable that the food found in the oysters during the latter part of the ebb was that ingested during the late stage of the flood, four to six hours prior to examination.

In general, the observations made on 160 oysters on August 21, 1945, showed that the oysters of Long Island Sound fed very actively during the ebb, and that the relative quantities of food found in their stomachs during that period were at least equal to or even exceeding those recorded during the last hour of the preceding flood.

Although observations in Long Island Sound have demonstrated that there was no correlation betwen the stages of the tide and the quantities of food found in the

stomachs of the oysters, it was desirable to supplement the data already available with additional observations on the oysters living under ecological conditions rather different than those of Long Island Sound proper. An area in Milford Harbor near the dock used for laboratory needs, was chosen for these observations. A large number of oysters living on the bottom near the dock made such an arrangement especially convenient.

Milford Harbor was selected because it was a typical example of a small partially inclosed body of water where extensive natural beds had existed. In recent times many of the beds were destroyed by overfishing, and the profile of the bottom was markedly changed by the dredging of a wide and deep channel. Nevertheless, the oysters quickly reestablished themselves in more shallow sections of the Harbor, and at present are quite common. A good setting of oysters regularly occurs in the Harbor, indicating that the conditions are favorable for the propagation of these mollusks. Usually changes in the temperature and salinity of the Harbor water during the tidal cycle are more pronounced than in Long Island Sound proper, where both these factors remain very steady (Loosanoff and Engle, 1940).

The observations consisted in examining the stomach content of the oysters at hourly intervals throughout a 24-hour period. The samples, each composed of six individuals, were suspended one day prior to the beginning of the examination in baskets made of 2-inch mesh poultry wire. All the baskets were kept at the same depth, namely, one foot below the mean low water line. They were separated from each other by a distance of approximately one foot and, therefore, the removal of any of the baskets did not disturb the oysters of the other containers. The experiment continued from 7:30 A.M. of July 27 until 8:00 A.M. of July 28, covering three low and two high water stages, and including periods of light and darkness (Table III). During this period the temperature of the water ranged from 22.0 to 25.0° C., the salinity fluctuated between 22.68 and 28.44 parts per thousand, and the pH from 7.7 to 8.7.

The data obtained indicated that during the two flood and two ebb periods covered, the majority of the oysters contained large quantities of food. Of the total number of 150 oysters examined 86 per cent belonged to that group. This figure closely approached that for the Long Island Sound oysters where 82 per cent were found to possess large quantities of food (Table I). Only 4 per cent of the Milford Harbor oysters were found with empty stomachs, the figure being too low to suggest that large groups of the oyster population ceased feeding for appreciatively long intervals during the period of observation.

More detailed studies of the data given in Table III do not offer sharply defined evidence which would lead to the conclusion that the oysters collected during the flood contained more food than those collected at ebb, or vice versa. Although it is true that all but one oyster collected during the second flood period, extending from 8:40 P.M. to 1:58 A.M., contained large quantities of food, virtually the same observations were made during the preceding period of ebb when 32 out of 36 oysters showed full stomachs. In each case only one oyster with an empty stomach was found. If the condition of the oysters during the first flood period (8:30 A.M. to 1:30 P.M.) is compared with that of the oysters examined during the second ebb period (3:00 A.M. to 8:00 A.M.), it will be found that in each case 28 individuals showed large, and 6 showed small quantities of food, while 2 oysters were with empty stomachs.

TABLE III

Temperature, salinity and pH of the water, and relative quantities of food in stomachs of oysters examined at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945. Each sample of oysters consisted of six individuals.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food			Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent						Large	Small	Absent
Low water	7:30 A.M.	24.2	22.68	7.7	6	0	0								
Flood								Flood							
1st hour	8:30 A.M.	24.0	27.74	7.7	4	2	0	1st hour	8:45 P.M.	24.5	24.40	8.7	6	0	0
2nd hour	9:30 A.M.	23.2	26.59	8.0	3	2	1	2nd hour	9:45 P.M.	23.9	25.73	8.5	6	0	0
3rd hour	10:30 A.M.	23.3	27.65	8.0	6	0	0	3rd hour	10:45 P.M.	23.3	26.00	8.5	6	0	0
4th hour	11:30 A.M.	23.0	28.44	8.0	3	2	1	4th hour	11:40 P.M.	23.3	26.00	8.5	5	0	1
5th hour	12:30 P.M.	23.1	27.65	8.1	6	0	0	5th hour	12:45 A.M.	23.1	26.54	8.5	6	0	0
High water	1:30 P.M.	23.1	26.33	8.2	6	0	0	High water	1:58 A.M.	23.0	26.27	8.5	6	0	0
Ebb								Ebb							
1st hour	2:30 P.M.	23.2	27.65	8.2	6	0	0	1st hour	3:00 A.M.	23.0	26.00	8.5	5	0	1
2nd hour	3:30 P.M.	23.0	28.51	8.3	6	0	0	2nd hour	4:00 A.M.	23.1	26.27	8.5	6	0	0
3rd hour	4:30 P.M.	23.4	26.39	8.3	4	2	0	3rd hour	5:00 A.M.	23.3	26.27	8.4	4	2	0
4th hour	5:30 P.M.	23.9	26.13	8.3	5	0	1	4th hour	6:00 A.M.	23.0	25.73	8.1	3	2	1
5th hour	6:30 P.M.	24.9	24.52	8.1	5	1	0	5th hour	7:00 A.M.	23.2	25.47	8.1	5	1	0
Low water	7:40 P.M.	24.7	24.13	8.4	6	0	0	Low water	8:00 A.M.	23.4	25.47	8.0	5	1	0
								Total					129	15	6
								Per cent					86	10	4

Perhaps it should be emphasized that among the 18 animals collected at the three low water stages (Table III) all but one oyster had large quantities of food in their stomachs.

Studies of the same nature as those made on July 27 and 28 were performed again on August 9. However, in the latter case observations were made only during one flood and one ebb stage covering a period of about 12 hours. The results of the observations are incorporated in Table IV together with data on the water temperature, salinity and pH recorded at the collection of each sample. The results showed that more oysters with large quantities of food were found in the group collected

TABLE IV

Relative quantities of food in oysters examined at hourly intervals during a 12-hour period in Milford Harbor on August 9, 1945. Each sample composed of 6 oysters. Temperature, salinity and pH are indicated for each stage of the tide.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent
Flood							
1st hour	7:55 A.M.	22.2	24.99	7.9	0	5	1
2nd hour	8:55 A.M.	21.9	25.72	7.9	1	5	0
3rd hour	9:55 A.M.	21.6	25.72	7.9	3	3	0
4th hour	10:55 A.M.	21.6	25.99	7 9	3	1	2
5th hour	11:55 A M	21.8	25.44	7.9	6	0	0
High water	12:55 P.M.	21.7	25.72	7.9	5	1	0
Total					18	15	3
Ebb							
1st hour	1:55 P.M.	22.0	25.72	8.0	4	2	0
2nd hour	2:55 P.M.	21.8	25.72	8.1	3	2	1
3rd hour	3:55 P.M.	22.0	25.72	8.1	6	0	0
4th hour	4:55 P.M.	23.9	25.44	8.1	6	0	0
5th hour	5:55 P.M.	24.4	23.84	8.0	4	2	0
Low water	7:05 P.M.	25.1	21.44	8.0	6	0	0
Total					29	6	1

during the ebb than among the animals examined during the flood. However, no significant difference was noted between the two groups in the number of oysters with empty stomachs.

In summarizing all our observations on the relative quantities of food present in the stomachs of the oysters during different tidal stages the conclusion may be formed that, as far as ingestion of food is concerned, the oysters of Long Island Sound and Milford Harbor do not show a definite preference either to ebb or to flood. During either stage the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally. The data obtained fail to lend any support to the unqualified opinion that the American oyster, *O. virginica*, takes relatively little food on the ebb tide.

Rate of water pumping in relation to tide

To determine the rate of water pumping of oysters and, therefore, the efficiency of their feeding at different stages of the tide an apparatus was constructed which permitted the measurement of the actual quantities of water passing through an oyster (Fig. 1). This apparatus was installed on the edge of the dock situated along the western shore of Milford Harbor where a swift tidal current flowed unobstruct-

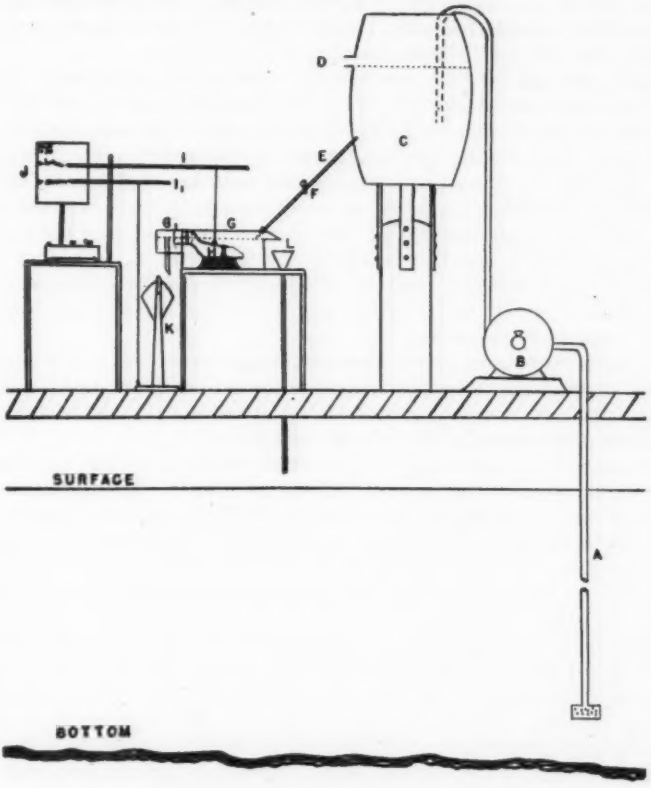

FIGURE 1. Diagram of the apparatus used in determining the quantities of water pumped by the experimental oysters. Description in text.

edly. The water supply was obtained through the hose, *A*, being pumped by the pump, *B*, into the storage barrel, *C*. The intake end of the hose, protected by a screen of large mesh always remained in the same position, six inches above the bottom. A constant water level in the barrel, *C*, was maintained by allowing the excess water to escape through the overflow outlet, *D*, to which a length of hose was attached. The capacity of the pump was such that the water in the storage barrel

was renewed every 4 or 5 minutes. Thus, the experimental oysters were receiving water, which was changing parallel with the changes of the tide.

The water was fed to the experimental oysters through the tube, *E*, provided with a flow-adjusting cock, *F*. The constant level oyster chamber, *G*, contained the oyster, *H*, the excurrent side of which was covered with a rubber, cone-shaped apron which conducted the water pumped by the oyster into the smaller chamber, G_1. Moore (1908) and Nelson (1936) were the first to apply the rubber apron, while Galtsoff (1926) devised and began to use the chamber. A string glued to the upper shell of the oyster was attached to the counter-balanced lever, *I*, which recorded every movement of the shell on the kymograph, *J*.

The water pumped by the oyster into the chamber, G_1, overflowed through the glass tube standpipe into the tripping vessel, *K*, of known capacity. When the vessel was filled with the water pumped by the oyster it tripped over, emptying its contents, and at the same time striking a string attached to the lever, I_1, which made a mark on the kymograph, *J*. Thus, each tripping was recorded, and because the capacity of the vessel was known, the quantity of water pumped by the oyster during certain intervals could be ascertained easily. The excess water entering the chamber, *G*, but not utilized by the oyster flowed out through the outlet, *L*. The part of the experimental apparatus containing the oyster chambers and the kymograph was kept in a small shed to protect the oyster from the effects of the sun and from possible disturbances caused by the wind.

The oysters were placed in the apparatus usually from one to two hours before high or low water. This time was allotted to the oysters to open their shells and to begin pumping water at a normal rate. This introductory period was not included in the analysis of the pumping activities of the oysters.

At the end of the introductory period, which usually coincided with the high or low water stage, observations were carried on for 12 or 13 hours, covering one ebb and one flood stage. In this manner records of 27 oysters were obtained. Fortunately, in almost all cases the oysters remained open continuously throughout the period of exposure. The experiments were conducted at temperatures ranging from 19.1 to 25.8° C., a range considered very favorable for the pumping activities of oysters (Galtsoff, 1928).

In analyzing the kymograph records obtained in the course of these studies it was found convenient to divide each flood or ebb period into six hourly subperiods. However, since the ebb and flood periods of Milford Harbor are not usually of exactly 6-hour duration, the data for the last hour of each stage had to be arrived at by determining the quantity of water pumped by the oyster from the end of the fifth hour until the change of the tide and then calculated for a 60-minute period.

Analysis of the data showed that, in general, the rate of pumping of the oysters during the flood stages was somewhat slower than that during the ebbing periods (Table V). However, because of significant differences in the rate of pumping shown by individual oysters within the same hour of a tidal period, and after considering all the aspects of the data secured the opinion was formed that the oysters of Milford Harbor feed actively at all stages of the tide, and that the rate of feeding during the ebb is at least equal to and sometimes may be even more rapid than during the flood.

In Milford Harbor the strongest tidal currents occur in the middle of the period between the high and low water stages. This period, therefore, corresponds to the

third and fourth hours of each stage. There is no evidence, nevertheless, that during this period the pumping of the oysters was more energetic than during the preceding or successive periods (Table V). It is of interest to note, however, that during the two last hours of the ebb the rate of pumping was somewhat accelerated.

The rapid rate at which many of the experimental oysters pumped water during ebb is well demonstrated in the photograph of the kymograph record showing the rate of pumping and shell movement of one of the experimental animals (Fig. 2). The period of observation lasted from 9:34 A.M. until 10:13 P.M., September 12, covering one complete flood and ebb period. Each vertical line of the lower record was made by the tripping vessel (Fig. 1), the capacity of which was 255 cc. Examination of the lower part of the record, which, incidentally, was made with the help of a dissecting microscope, a method always employed when the pumping was rapid and the marks on the kymograph were made close to each other, provided the following information: the minimum quantity of water pumped by the oyster during a single hourly period of the flood was 4080 cc., and the maximum, 20,655 cc. The

TABLE V

Mean of quantities of water (in cubic centimeters) pumped by oysters during each hour of the flood and ebb periods. The data are based on the kymograph records of 27 oysters, Milford Harbor, Summer of 1945.

Stage of tide	Mean	Stage of tide	Mean
Flood		Ebb	
1st hour	15,952±1,214	1st hour	15,384± 787
2nd hour	14,411±1,578	2nd hour	15,962± 830
3rd hour	12,875±1,427	3rd hour	16,186±1,021
4th hour	12,470±1,389	4th hour	17,458±1,195
5th hour	13,438±1,130	5th hour	17,916±1,063
6th hour	14,131±1,040	6th hour	17,577± 944

average hourly rate of pumping for the entire flood period was 13,260 cc. For the ebb period these figures were 15,810, 23,715, and 20,244 cc. respectively.

Examination of other records of the same series revealed that during the ebb some of the oysters averaged from 25 to 27 thousand cc. per hour, while the maximum rate of pumping in some instances ranged from 31 to 34 thousand cc. per hour. Having in possession a large number of kymograph records of this nature one may well be inclined to disagree with the opinion that the American oyster is relatively inactive during the outgoing stage of the tide.

In connection with this discussion an interesting deduction can be made concerning the efficiency of the pumping mechanism of oysters. The average weight of the oyster meat removed from the shell the length of which is 4 inches is approximately 20 grams and its volume is usually not more than 20 cc. This organism, nevertheless, is capable of pumping 30 thousand cc. or more of sea water per hour. In other words, the volume of water passing through the oyster gills in one hour may be more than 1500 times greater than the volume of the oyster's body, a fact well attesting the efficacy of the feeding mechanism of this mollusk.

FIGURE 2. Photograph of kymograph record showing the shell movements (upper line) and rate of pumping (lower line) of oyster no. 19 obtained during the flood and ebb stages in Milford Harbor on September 12, 1945. Each vertical line of the lower record designates emptying of the tripping vessel (Fig. 1) of 255 cc. capacity. *L. W.* and *H. W.* indicate the low water and high water stages respectively.

Opening and closing of shells in relation to tide

Experiments for determining the presence or lack of correlation between the opening and closing of the oyster shells in relation to the tidal stages were also conducted in the Summer of 1945. The method used was the same as that successfully employed in the studies of the shell movements of hard shell clams, *Venus mercenaria*, and of the edible mussel, *Mytilus edulis* (Loosanoff, 1939, 1942). A description of this apparatus has already been published in full (Loosanoff, 1939) and, therefore, need not be repeated. It will be sufficient to say that this apparatus permitted registering on the Foxboro recorder of every shell movement of oysters kept under virtually natural conditions on the platform installed on the small oyster bed existing on the bottom of Milford Harbor. Even at low tides the experimental oysters were covered with at least 2 feet of water.

The area where the apparatus was installed was subjected to strong tidal currents which, during the spring tides, attained a velocity of 1.2 feet per second on the flood, and 1.5 feet per second on the ebb. During the neap tides, however, the velocity of the flood current was only 0.8 foot per second, and that of the ebb current, 1.3 feet per second. The mean range of tide in Milford Harbor is 6.6 feet, but during the spring tides high water occasionally reaches the 9-foot mark. In Milford Harbor, the same as in Long Island Sound, the strongest tidal currents occur in the middle of the period between high and low water. The slack usually coincides with the time of the high and low water stages.

Usually the records of two oysters were taken simultaneously—each record covering a 24-hour period. The temperature of the water was also continuously registered by Brown's recording thermometer which was installed near the shell movement recording apparatus, the bulb of the thermometer being only 5 inches away from the experimental oysters. The temperature range extended from 17.0 to 28.0° C., averaging approximately 22.0° C. Altogether 64 records of 18 different oysters were obtained in the course of these observations.

That the conditions in the Harbor were favorable for the existence of the mollusks was well shown by the growth of the young oysters which attached themselves to the concrete base of our apparatus used for studying the shell movements of the experimental animals (Fig. 3). The attachment, or, as it is more commonly called, the setting of the oysters took place early in August, soon after the apparatus was first placed in the water. The examination made late in September showed that the young oysters grew very well, regardless of the fact that the base was often removed from the water for short periods to change the experimental mollusks. This rapid growth of the young oysters, as well as their generally good condition, indicated that the experimental animals, the shell movements of which were recorded, were also subjected to favorable environment.

Analysis of 64 complete records showed that on an average the oysters remained open 22 hours and 39 minutes, or 94.3 per cent, and closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period. Our figure for the duration of openness of the oyster shells is quite close to that of Nelson (1921) who, basing his conclusions on the records of 3 oysters, found that these animals remained open on an average of 20 hours per day, but is much higher than that offered by Galtsoff (1928) who found that the average period the oysters remain open is 17 hours and 7 minutes per day. However, the difference between Galtsoff's figures and ours may be explained on

the basis that his observations were made on the oysters placed in the aquarium, while our animals were kept on the bottom of the Harbor, where conditions were different from those of the laboratory. Nevertheless, Galtsoff expresses the opinion, which coincides with ours, that oysters have a tendency to keep the shells open as long as possible.

Further statistical studies of the data collected showed that during the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the

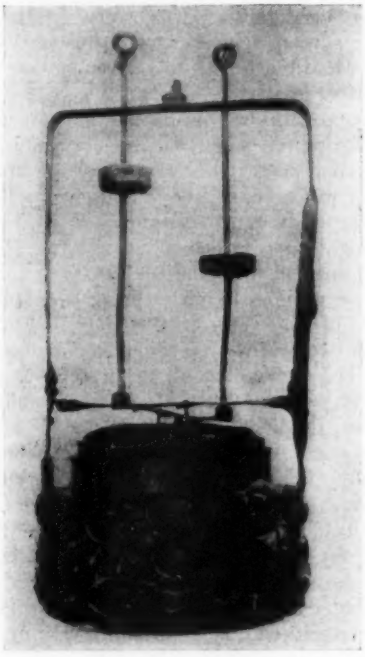

FIGURE 3. Photograph of the base of the instrument employed to record the shell movements of the oysters kept on the bottom of Milford Harbor. Note the healthy and vigorously growing young oysters attached to the sides of the base. Large experimental oysters imbedded in small concrete blocks can be seen on the top of the base.

time, whereas during the ebb periods the shells were open 95.2 per cent. Obviously, the difference between the two figures is rather insignificant, indicating that the behavior of the oysters is quite similar during the opposite stages of the tide.

Another trait of similar behavior of the oyster during the two different tidal stages was indicated by the fact that almost an equal number of records showing that the animals kept their shells open 100 per cent of the time was obtained for each stage. Thirty-three such records were made during the flood periods, and 32, during the ebb

Feeding in relation to time of day

While conducting these studies the opportunity presented itself to verify the statements that the feeding of oysters is considerably slowed down (Nelson, 1921, 1923) or entirely ceases (Orton, 1929) at night and in the early morning. The material for studying this problem was available from all three phases of our investigation, namely, observations on the stomach content of oysters, rate of pumping, and opening and closing of the shells.

Examination of the stomach content of the oysters made at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945, has shown that during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. (E.W.T.), the oysters contained as much food as during the hours of light (Table III). During the largest part of the period of darkness, until 5:00 A.M., almost all the oysters contained large quantities of food. The percentage of individuals with full stomachs during this period would compare favorably with that of the animals examined during the daytime. Numerous other examinations of the stomach content of the Milford Harbor oysters made between 1:00 and 6:00 A.M. during the Summer of 1945 also showed that the majority of the oysters had full stomachs. Obviously, these observations do not offer any support to the conclusion that oysters do not feed at night.

The results of the experiments in which the rate of pumping of the oysters was determined also contradict the above mentioned conclusion. Observations performed at night showed that the oysters remained active and fed very vigorously. The results of the experiment conducted during the night of July 27–28 were especially significant because in that case the period of late night coincided with the ebb (Fig. 4). Thus, the effects of two presumably unfavorable factors were combined. Nevertheless, as is shown in Figure 4, both experimental oysters remained open and continued to pump water at a very rapid rate during the entire period of darkness. When one of the animals closed at about 6:30 A.M. it already was one hour after sunrise. During the period of darkness the average rate of pumping of oyster no. 92 (upper record) was 17,934 cc. per hour, and the maximum, 19,272 cc. per hour. For oyster no. 98 (lower record) these figures were 24,622 and 30,600 cc. respectively.

Because this experiment was of a 24-hour duration data were also available on the rate of pumping of the same two oysters in daylight. The average rate of pumping of oyster no. 92 during that period was found to be 12,684 cc. per hour, and the maximum, 19,053 cc. per hour. For the second oysters the corresponding figures were 24,565 and 31,875 cc. Thus, in this case, the same as in the case of studies of the stomach content of the oysters, the conclusion may be offered that oysters feed actively during darkness. Furthermore, the average rate of pumping at night was not lower than during the daytime.

A series of supplementary experiments on the effect of light and darkness upon the rate of pumping also failed to show a significant difference in the behavior of oysters. These experiments were conducted in a dark room, where an apparatus of the type shown in Figure 1 was installed. Three or four oysters were tested simultaneously. During the period of light the oysters were illuminated with small floodlights. The intensity of light at the surface of the oysters, as determined by the Weston photronic exposure meter, was in excess of 1000 candles per square foot.

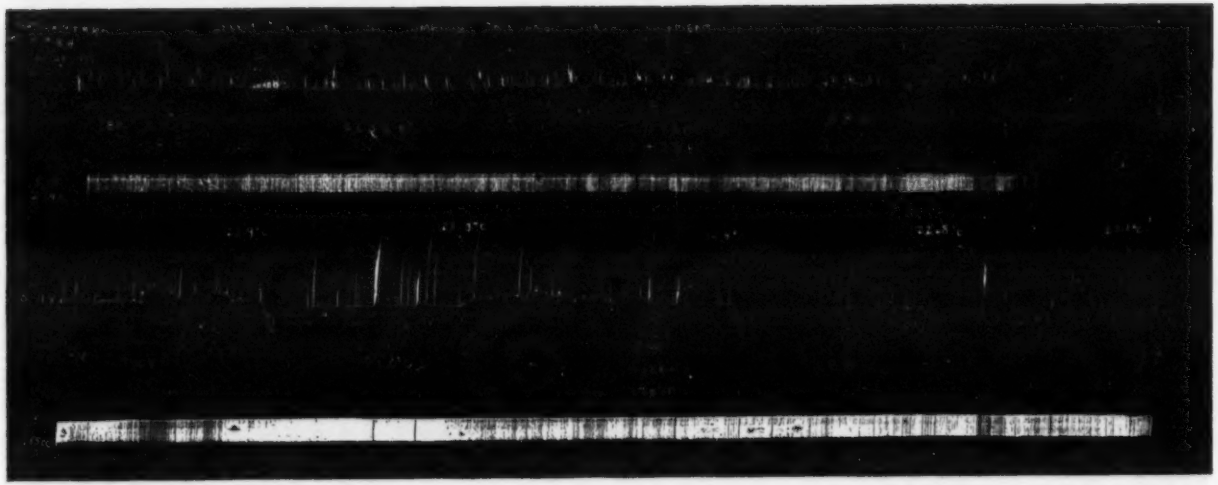

FIGURE 4. Photograph of the kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters during the period extending from 7:50 P.M., July 27 until 8:17 A.M. July 28, 1945. Each vertical mark of the second line designates emptying of the tripping vessel of 219 cc. capacity, while each mark of the fourth line shows the dumping of 255 cc. of water. The records show that both oysters fed very actively during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. *L. W.* and *H. W.* indicate the low water and high water stages respectively.

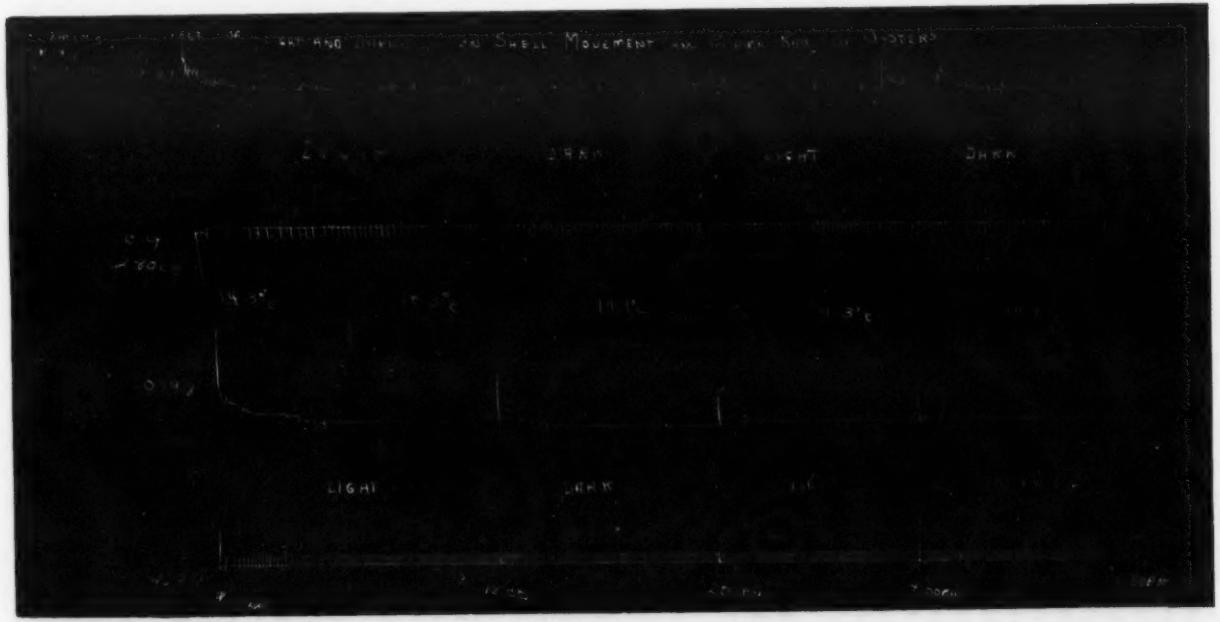

FIGURE 5. Photograph of kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters exposed to alternating periods of light and darkness. Each vertical mark of the second line designates the emptying of the tripping vessel of 280 cc. capacity, while each mark of the fourth line shows the dumping of 237 cc. of water.

The oysters were kept in the apparatus for approximately 9 hours (Fig. 5). During this time the mollusks were exposed to two periods of light and two of darkness. However, the initial period, of approximately 3 hours, was not included in the analysis, as this time was given to the oysters to become accustomed to the experimental condition. To equalize the data half of the experiments began with a period of light, and the other with that of darkness. Altogether 30 records of 10 different oysters were obtained. Analysis of the data showed that the mean hourly rate of pumping during the periods of light was 5350 ± 445 cc. For the periods of darkness this figure was 5036 ± 385. Thus, the rate of pumping during periods of light closely resembles that during periods of darkness.

Finally, the data on the same subject were taken from our 64 daily records of the shell movements of oysters. Analysis of these data showed that on an average the shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the periods of darkness. The difference of less than one per cent is not considered as significant in this case, and, therefore, it may be concluded that in our experiments no correlation was found between the periods of closure of the shells and darkness. These results are in agreement with those of Galtsoff (1928) who in his article disagrees with Nelson's (1921, 1923) conclusion that the period of darkness, between 11:00 P.M. and 4:30 A.M., should be considered as a time of rest for oysters.

Discussion

The advocates of the opinion that oysters considerably reduce their feeding activities during the periods of ebb failed to suggest in their publications any factors which could be considered as responsible for the change in the behavior of the mollusks. It could be easily understood that in some areas, where the periods preceding and coinciding with the low water stage are accompanied by distinctly unfavorable changes in the environment, the oysters would temporarily slow down or even cease feeding. For example, a sharp decrease in salinity could compel the oysters to be relatively inactive. However, according to Nelson (1921) the reduction in salinity was not the cause. This conclusion is based upon his statement that in Huey's Creek, where the experiments were conducted, the periods of complete cessation or of the commencement of feeding, although showing a definite correlation with the stage of the tide, occurred independently of the changes in the density of the water, because such changes were usually of small magnitude.

Changes in the turbidity and temperature of the water were also considered as unimportant by Nelson (1923), who concluded that "The rate of filtration of water during any given period of time, as deducted from the rapidity and extent of ejections of accumulated sediment from the mantle cavity, may vary widely independently of the temperature and the turbidity of the water." In the same article Nelson also stated that "No correlation could be shown between the food content of the water and the periods of inactivity of the oyster." All these conclusions were based upon the experimental data first reported by Nelson in 1921. Thus, according to that author, neither changes in salinity or temperature, nor changes in the turbidity or quantity of food present in the water affected the rate of feeding of oysters. Yet, because of some undetermined factors these mollusks fed much less actively during the ebb stages.

In discussing Nelson's (1921) work it is necessary to mention that his experiments were devised to study the shell movements of the oysters, but not the rate at which these mollusks were filtering water through the gills, i.e., feeding. Only the shell movements of the oysters were recorded on the kymograph, while no data were obtained on the quantities of water pumped by the oysters during the different tidal stages. Obviously, no definite conclusions could be formed concerning the latter subject because of the almost complete lack of experimental evidence regarding this matter.

Nelson's method of interpreting the data should also be mentioned. In analyzing his material on shell movement of the oysters Nelson (1921) takes into consideration only the numbers of openings and closures of the shells during the different tidal periods. This method has already been criticized by Galtsoff (1928) who stated that "The examination of the number of closures and openings occurring during a given period of time does not convey a true idea of the activity or inactivity of the oyster. A better understanding can be gained by counting the number of hours the oyster was closed or open during a given period of a day." Obviously, Galtsoff's suggestion is well founded.

Our observations and experiments supplied the evidence that the oysters of Long Island Sound and Milford Harbor fed actively during the flood and ebb periods, and that during the ebb their feeding was often more energetic than during the flood. Observations of this nature could probably be made in many other bodies of water where changes in the tides are not accompanied by pronounced ecological changes. This conclusion appears to be logical because it is quite improbable that, if other conditions of the environment remain favorable, a change in the direction of the tidal current alone would affect the oysters. It is, to a certain extent, supported by our experiments in which pairs of oysters, employed in our studies of the shell movements, were always placed so that the hinges of their shells pointed in opposite directions. Thus, while the gills of one oyster faced the flood, the gills of the other animal were turned away from the direction of the current. Yet, no difference suggesting that an oyster in a certain position was more active on the flood or ebb was generally noted.

No correlation between the rate of water pumping of the oysters and the time of day was demonstrated by our experiments. Neither was it found that the duration of the opening and closure of the shells was affected by periods of light or darkness. In nature many oyster beds are located at a considerable depth. Very often the water flowing over the beds contains large quantities of suspended matter which stop the penetration of a large quantity of light before it reaches the bottom on which the oysters live. These oysters, therefore, normally exist in near-darkness even during very strong daylight. It is very doubtful that if other conditions remain favorable, the slight change in the intensity of illumination caused by the approach of night could have such a pronounced effect on the oysters that they would either begin to feed at a much slower rate or stop feeding entirely.

If the rate of feeding of oysters were markedly decreased during the nights and during the ebb periods, the existence of these mollusks would be under a rather unfavorable condition. Because the periods of darkness are often followed by ebb, there would be times when the feeding activities of the oysters would be continuously depressed for a period of approximately 18 hours. This condition would occur in September and October when the nights become long. However, it is a

well known fact that during these two months the oysters of our waters undergo very rapid improvement in condition storing large quantities of glycogen in their bodies. Naturally, such an improvement could not be possible if, during this time of the year, the oysters had to exist under the conditions compelling them to be relatively inactive during approximately 12 hours of darkness and also during the 6 hours of ebb, a total of 18 hours per day.

As may be seen from this discussion, our conclusions regarding the activities of oysters during ebb and during periods of darkness do not agree with those of Nelson and Orton (ref. cit.). However, as Dr. Nelson suggests in recent personal communication with the senior author, the cause of the divergence may be attributed to the marked reduction of the pH during ebb and at night in the waters where Nelson's experiments were conducted. During the outgoing tide those areas received large quantities of swamp water which noticeably lowered the pH. Also, according to Nelson "These waters are but slightly buffered; hence at night with the respiration of algae and of animals and decomposition the water may become acid by morning." Such changes are indicated in one of Nelson's reports (1924). The conditions, however, are different in other basins, such as in many sections of Chesapeake Bay (Loosanoff, 1932) and Long Island Sound (Loosanoff and Engle, 1940) where the pH does not closely approach the neutral point.

In general, our experiments have shown that under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor and Long Island Sound. Although the differences in the behavior between the individual oysters are of considerable magnitude, these mollusks, nevertheless, appeared to be feeding all or most of the time their shells remained open which, with a temperature range from 17.0 to 28.0° C., was approximately 94 per cent of the total time.

In presenting the final conclusions it should be once more emphasized that we do not interpret our results as applicable to all oyster growing areas of this coast. While our observations hold true for the areas where the experiments were conducted, and also, probably, for the waters where the ecological conditions resemble ours, it is realized that in other basins, where during ebb the oysters are exposed to unfavorable environment, different conditions may prevail. Nevertheless, the material presented in this article clearly indicates that the conclusions of Nelson (ref. cit.), which, no doubt, are representative for Huey's Creek, should not have been generalized and presented as applicable to the American oyster as a species (Orton, 1929).

Summary

1. Examination of approximately 1400 oysters collected during the different tidal stages in Long Island Sound and Milford Harbor failed to show any definite period when the stomachs of these mollusks displayed absence of food.

2. During all hours of the flood and ebb, including the low water period, the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally.

3. The relative quantities of food found in the oyster stomachs during the ebb period were at least equal to or sometimes even exceeded those recorded during the flood.

4. Analysis of the kymograph records of the rate of water pumping by the oysters showed that they fed very actively at all stages of the tide, and that the rate of feeding during ebb was at least equal to or sometimes even more rapid than during the flood stage.

5. During the ebb some of the oysters pumped on an average of 25,000 to 27,000 cc. of water per hour, while the maximum rate of pumping in some instances ranged from 31,000 to 34,000 cc. per hour.

6. The efficiency of the feeding mechanism of an oyster may be well attested by the fact that the volume of water passed during one hour through the oyster gills may be more than 1500 times greater than the volume of the oyster's body.

7. Within the temperature range of 17.0 to 28.0° C. the oysters remained open on an average of 22 hours and 39 minutes, or 94.3 per cent, and were closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period.

8. During the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the time, whereas during the ebb periods the shells were open 95.2 per cent.

9. During the periods of darkness the percentage of oysters with full stomachs was comparable to that of the individuals examined during the day time.

10. During darkness the oysters were found feeding very actively. The average rate of pumping at night was not lower than during the daytime.

11. The shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the period of darkness. No correlation was found between the periods of closure of the shells and darkness.

12. Under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor. These mollusks were found to be feeding all or most of the time when their shells remained open.

13. The results of this investigation do not lend any support to the generally accepted theory that the American oyster does not feed late at night and in the early morning, and is relatively inactive on the ebb tide.

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AUTOSOMAL ELIMINATION AND PREFERENTIAL SEGREGATION IN THE HARLEQUIN LOBE OF CERTAIN DISCOCEPHALINI (HEMIPTERA)

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Introduction

It has been known for some time that the various lobes in the testis of many species of pentatomid Hemiptera show constant differences in the size of their cells. Bowen (1922a and b) who investigated this condition most recently, concluded that it is attributable mainly to differences in the volume of cytoplasmic elements. That is a finding which I can only confirm; if differences in the volume of chromatin exist they must be very small. However in some species there is a testicular lobe whose cells differ from those of other lobes not only in size but which has also evolved an entirely novel process of maturation. The main features of this maturation are in several instances almost fantastic in character, and the evolution and constant occurrence of such "harlequin" lobes is a matter of some interest.

It should be emphasized that we are dealing here not with accidental or sporadic occurrences. In the species concerned the harlequin lobe is found in each testis of every male. Moreover for any given species it is always a certain and very definite lobe that is thus characterized (in the Discocephalini here treated it is the fifth) and hence it is clear that its development involves conditions that are fundamentally and firmly established in the species as it is now constituted.

Harlequin lobes have so far been encountered in three species of Loxa, a species of Mayrinia (Schrader, 1945a and b), and in a species of Brachystethus (Schrader, 1946). In the last named the departure from a normal meiosis lies primarly in the autosomes which are shunted out of the spindle in both divisions; in Loxa and Mayrinia the aberrancy takes the form of amitosis and fusion in the spermatocytes, resulting in a highly variable heteroploidy. The meiotic anomalies of Brachystethus and Loxa thus appear to be in no way related and yet it seems only natural to assume as a working hypothesis that the evolution of harlequin lobes involves similar basic conditions in all the species involved.

It is likely that further investigations will discover that harlequin lobes are present in a great many species. To the five species mentioned above and the three taken up in the present paper may be added at least three further species which I have not as yet fully analyzed—a total of eleven. Taxonomically speaking, these species cover a wide range. Loxa and Mayrinia represent typical genera of the tribe of Pentatomini; Brachystethus is so closely related to the Edessini as to furnish almost a "bridging" genus between that tribe and the Pentatomini; and the Discocephalini constitute a tribe so distinct from the other pentatomid tribes that it has sometimes been elevated to the rank of a subfamily (Lethierry and Severin, 1896).

Conditions in the females of all these species are still unknown, except as they were used in all instances to check the identification of the sex chromosomes in the males. Cytologists need hardly be told that this gap in our knowledge is due mainly to the technical difficulties that render a study of meiosis in the egg so onerous a task.

I should like to point out again as I have done in my study of Brachystethus, that the investigation of the harlequin lobe is made under the almost ideal conditions of a natural experiment. The adjoining lobes of the same testis are perfectly normal and serve continually as a control; frequently the normal and aberrant cells can be studied in one and the same field of the microscope.

Material and Methods

The Discocephalini investigated are: *Mecistorhinus melanoleucus* Westwood (one male from Panama); *Mecistorhinus tripterus* Fabricius (four males and two females from Costa Rica); *Mecistorhinus sepulcralis* Fabricius (one testis each from eight different males and the ovaries from one female, all from Piracicaba, Brazil); *Neodine macraspis* Perty (five males from Costa Rica); and *Platycarenus notulatus* Stål (three males and one female from Costa Rica). The last named species has no harlequin lobe and is only briefly mentioned in the following pages.

My thanks are due to the eminent hemipterist, Mr. H. G. Barber, who identified all the species of Mecistorhinus. To Professor S. de Toledo Piza of the University of São Paulo, Brazil, I am deeply indebted for the material of *Mecistorhinus sepulcralis*.

Fixation was made in either Bauer's convenient modification of Allen's Bouin or in Sanfelice. As in all my recent studies of mitosis, I have employed three staining methods. The Feulgen technique is indispensable as a test for chromatin; gentian violet (in Smith's modification of Newton's method) is often very useful for a study of the detailed structure of the chromosomes but even when combined with erythrocin is not an efficient stain for the spindle apparatus in pentatomids; whereas Heidenhain's hematoxylin remains beyond all comparison the best means for bringing out asters, centrioles and spindle fibers. As noted above, female material was studied only to check identification of the sex chromosomes in the male.

In all the Discocephalini where a harlequin lobe occurs, it is the fifth of seven lobes in each testis. In every case it is two or three times as voluminous as any other lobe although its spermatocyte cells after the leptotene stage are smaller than those of the rest of the testis. The fourth and sixth lobes which flank it on either side carry exceptionally large but otherwise normal cells, whereas the remainder conform to more orthodox proportions.

In the following pages the different species are taken up separately, the detailed analysis of *Mecistorhinus melanoleucus* being followed by briefer comparative accounts of the other forms. As far as possible, the interpretative treatment is relegated to the discussion that terminates the paper.

Mecistorhinus melanoleucus

Normal lobes

Except for certain features which are pertinent to an analysis of the peculiarities of the harlequin lobe, no detailed account of the spermatogenesis in the six normal

lobes need be given. It conforms closely in its general course to that which has often been described in other pentatomids.

The diploid set of fourteen chromosomes is marked by one exceptionally large pair of autosomes. This pair stands out almost as conspicuously as does the X chromosome of Protenor. Here however, the sex chromosomes are relatively small, the Y being the smallest member of the complement and the X little if any larger than the smallest of the autosomes (Fig. 2).

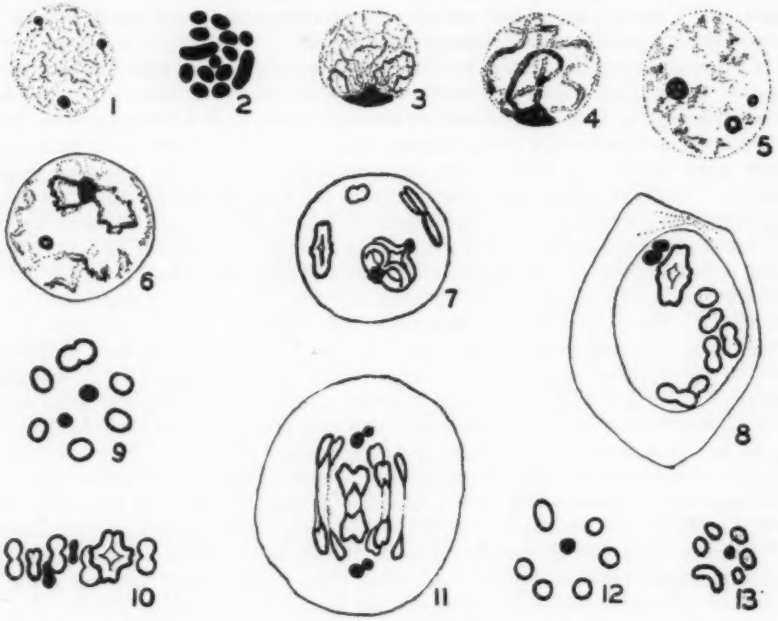
One of the exceptional features lies in the heteropycnosis that marks not only the sex chromosomes but also certain of the autosomes. Already in the early generations of the spermatogonia there are from three to five heteropycnotic bodies that stand out prominently in the resting phase (Fig. 1). There is no prochromosome stage intervening between the last spermatogonia and the leptotene stage of meiosis. In the leptotene and synaptic stages the heteropycnotic bodies are usually aggregated in a single mass on the nuclear periphery and it is at this locus also that the fine leptotene threads come together in a bouquet formation (Fig. 3). But in some cells there are two heteropycnotic bodies during these stages, the second and smaller one usually lying at some distance from the first and not necessarily at the periphery. The advent of the pachytene and diplotene stages sees little change (Fig. 4) in these conditions of heteropycnosis and it is only when they in turn give way to the confused stage that the single heteropycnotic aggregate is dissociated again. In this peculiar phase when staining conditions and despiralization temporarily convert most of the chromosomes into pale and flocculent threads there may again be three, four, or five heteropycnotic bodies (Fig. 5). This variation in number would seem to indicate that the mutual and nonspecific attraction that brings heteropycnotic chromosomal bodies together at certain stages is not very strong and it is likely that accidents of position determine these numbers to some extent.

In early diakinesis, as the chromosome threads again become definite in outline, the topographic relationships are once more open to analysis. Now the great majority of cells show only two heteropycnotic bodies, one of which becomes less and less conspicuous as the threads shorten and condense [1] while the larger one is seen to be intimately associated with the big bivalent (Fig. 6). Somewhat later, when the paired chromosomes have assumed the typical cross and ring formations of late diakinesis this large heteropycnotic body has disappeared, but there are then two smaller bodies, one associated with each of the two spreading arms of the large bivalent (Fig. 7). There is no doubt about the identity of these bodies. The larger is the X and the smaller the Y, the two together constituting the larger heteropycnotic body or chromosome nucleolus of earlier diakinesis. The dissociation of this single nucleolus into its two components is perhaps due not only to the strains that attend the separation of the arms of the large bivalent but may be a part of the regular cycle that in other species also sees the reappearance of the separate sex chromosomes at this stage. What is more remarkable is their persistent union with the arms of the autosomal bivalent, a union which is not broken until shortly before metaphase.

The rather even peripheral distribution of the diakinetic bivalents disappears

[1] The present state of our knowledge concerning the changes in the chromosome during a complete mitotic cycle is still unsatisfactory. Almost certainly both coiling and nucleination are involved, but the relative importance of these two factors remains undetermined. For that reason the terms "condensation" and "diffuseness" are here used in a purely descriptive sense.

just before the disintegration of the nuclear membrane. They then lie helter skelter in the nucleus and may even come into contact with each other. It is at this time when the chromosomes are in the final stages of condensation that the two sex chromosomes sever their connections with the large tetrad, though their former as-



Mecistorhinus melanoleucus—Normal Lobe

FIGURE 1. Early prophase in spermatogonial cell; three chromatin nucleoli (Feulgen).
FIGURE 2. Spermatogonial metaphase; Y is smallest of the 14 chromosomes (Feulgen).
FIGURE 3. Leptotene stage (Feulgen).
FIGURE 4. Diplotene stage (Feulgen).
FIGURE 5. Confused stage (Feulgen).
FIGURE 6. Early diakinesis; XY nucleolus attached to large tetrad (Feulgen).
FIGURE 7. Late diakinesis; X and Y attached to separate arms of large tetrad (Hematoxylin).
FIGURE 8. Prometaphase; X and Y still close to large tetrad (Hematoxylin).
FIGURE 9. Metaphase I; polar view (Hematoxylin).
FIGURE 10. Metaphase I; side view (Feulgen).
FIGURE 11. Anaphase I; large tetrad lagging (Gentian violet).
FIGURE 12. Metaphase II; polar view (Hematoxylin).
FIGURE 13. Telophase II; 6 autosomes + Y (Feulgen).

sociation is frequently indicated by their close proximity to it (Fig. 8). It is this stage also that is marked by an elongation of the nucleus as a whole in the polar axis, a change that plainly involves interaction with the two centers located at the periphery of the cell.

About the meiotic divisions themselves, little need be said. Metaphase I is quite typical in its conformation, with the now separated X and Y usually in the center of a ring of six tetrads (Fig. 9). It is however worthy of note that the large bivalent is somewhat slower than the rest of the autosomes in its condensation and side views of the first equatorial plate still show it as a cross tetrad (Fig. 10). At anaphase the X and Y divide equationally and arrive at the poles before the rest of the chromosomes, whereas the large bivalent (often showing the tertiary split) lags in its division and is distinctly slower than the other autosomes in its anaphasic progress (Fig. 11).

The second division also witnesses some lagging on part of the large autosome, but this is not as striking as in the first division. The touch and go pairing of the X and Y occurs as usual, and in metaphase they line up in the spindle axis so that in polar views one is superimposed on the other (Fig. 12). They then separate to oposite poles and the spermatids receive the typical pentatomid complements of 6A + X and 6A + Y respectively (Fig. 13). The departures from the orthodox process of meiosis thus do not affect the results, which conform to the regular pentatomid scheme.

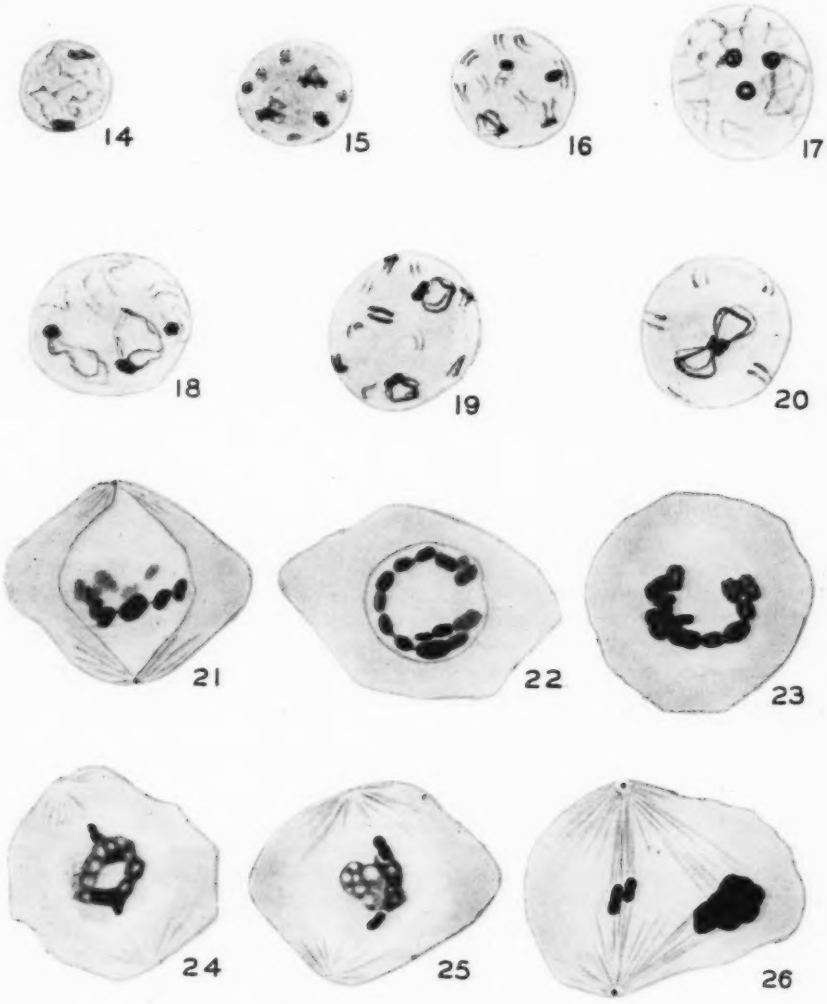
Harlequin lobe

Spermatogonia and meiotic prophases

The spermatogonial stages in the harlequin lobe differ in no discernible way from those of the normal lobes. Here too there are, in Feulgen preparations, from three to five heteropycnotic bodies in the resting phase and the succeeding stages closely parallel the normal course of events. As in the normal lobes there is no prochromosome stage. The meiotic leptotene duplicates that of the other lobes in the number and disposition of heteropycnotic bodies, but the chromosome threads do not seem to be as finely drawn out and delicate as they normally are. This difference however is too slight to furnish a secure basis for contrast (Fig. 14). Succeeding this stage the developments follow a path that diverges widely from the usual one.

There is neither a synapsis nor a pachytene stage. During the period in which these developments occur in the normal lobes, the chromosome threads of the harlequin lobe merely abandon their bouquet orientation and undergo a progressive condensation. As a result the nucleus then shows twelve somewhat loosely coiled autosomes and the two more condensed sex chromosomes, clear evidence that any sort of pairing that may have occurred unobserved prior to this time has now been abrogated (Fig. 15). The picture presented is a surprisingly close approximation of the prochromosome stage as it occurs normally in some Hemiptera, and in this respect has some resemblance to the conditions in the harlequin lobe of Loxa (Schrader, 1945b) where such a post-leptotene condensation is also encountered. But in Loxa there is a true prochromosome stage as well which occurs quite normally prior to the evolution of the leptotene threads. Both there and in Mecistorhinus no confusion is possible for not only does the true prochromosome stage occur much higher in the testis, but its nuclei are considerably smaller than are the ones here in question.

This post-leptotene condensation culminates in shortened, fuzzy chromosomes that show an equational split (Fig. 16), undoubtedly a condition corresponding to



Mecistorhinus melanoleucus—Harlequin Lobe

FIGURE 14. Leptotene stage (Gentian violet).
FIGURE 15. Post leptotene condensation; X and Y heteropycnotic (Feulgen).
FIGURE 16. Diplotene stage in univalents (Gentian violet).
FIGURE 17. Late confused stage (Feulgen).
FIGURE 18. Early diakinesis; sex chromosomes attached to separate large autosome (Gentian violet).
FIGURE 19. Mid-diakinesis; 12 univalents autosomes, with each large autosome combined with one sex chromosome (Gentian violet).

the diplotene stage of the normal lobes. Throughout this period two pairs of chromosomes are readily recognizable; they are the two large autosomes and the two heteropycnotic sex chromosomes. Each large autosome has its ends united so as to form a split ring, a configuration that very probably arises from the mutual attraction of its heteropycnotic terminal regions. The two heteropycnotic sex chromosomes show no such attraction at this time and usually lie well separated, evidence that heteropycnotic attraction is confined to certain conditions of the heterochromatin.

The confused stage which now intervenes, temporarily halts a further close analysis of progressive chromosome changes. The autosomes once more become diffuse and uncoiled and at the height of the stage stain very lightly. Usually three heteropycnotic bodies are present at this time, but there may be as many as five (Fig. 17). In the latter case the bodies are smaller, generally speaking, which would indicate that the variations in number are due to some vagaries in mutual attraction and aggregation. The two sex chromosomes and the ends of the two autosomes would account for six such bodies which suggests that some aggregation is nearly always present.

With the termination of the confused stage and the beginning of diakinesis, the individual chromosomes once more appear as such. There are then three heteropycnotic bodies and two of these are seen to be associated with the two large autosomes (Fig. 18). The third shows no such definite association and gradually disappears. In mid-diakinesis a more exact analysis of these conditions becomes possible. At this time there is a total of either eleven or twelve chromosomal bodies in every nucleus. When there are twelve, the two autosomes are quite independent of each other and may lie far apart. Each of them has a large chromatin nucleolus or heteropycnotic body attached to it at the place where the ends are still joined in ring formation (Fig. 19). When on the other hand there are only eleven bodies, these two chromatin nucleoli have come together, and through them the two large, ring formed autosomes have joined in a figure eight (Fig. 20). The two chromatin nucleoli represent the X and Y chromosomes and again, the most natural explanation of such configurations would seem to lie in the forces of heteropycnotic attraction; the heteropycnotic ends of the large autosomes are drawn together to form rings, and the heteropycnotic sex chromosomes later become attached to these regions and to each other for the same reason. It is rather strange that no case has been encountered in which both sex chromosomes have become joined to only one of the large autosomes, since nonspecific heteropycnotic attraction might be expected to give rise occasionally to such configurations. However, nuclei of this stage in which the chromosomes are open to a clear analysis are not common and

FIGURE 20. Mid-diakinesis; both sex chromosomes and both large autosomes in one combination (Gentian violet).

FIGURE 21. Equatorial ring side view (Hematoxylin).

FIGURE 22. Equatorial ring slightly later; polar view (Gentian violet).

FIGURE 23. Autosomes in precocious return to diffuse condition; X and Y still heteropycnotic (Gentian violet).

FIGURE 24. Formation of autosomal aggregate; X and Y heteropycnotic (Gentian violet).

FIGURE 25. Dissociation of X and Y from autosomal aggregate (Gentian violet).

FIGURE 26. Metaphase I; X and Y on middle spindle and autosomal aggregate displaced (Hematoxylin).

the fourteen examples which have been studied hardly constitute a sufficiently large number to justify the conclusion that they do not occur.

Prometaphase

Shortly before the breakdown of the nuclear membrane a significant reorientation of the chromosomes takes place. This is at about the time that the nucleus elongates toward the peripherally located centers. The chromosomes, still not fully condensed, then are shifted to the middle region between the centers and since they remain in close proximity to the nuclear wall and have lost the property of mutual repulsion, they tend to form a more or less circular row or chain in the equator. Some of the components of such chains may be in actual contact with each other, while others may be connected by Feulgen positive bridges or show no attachment at all (Figs. 21, 22, and 72). It is likely that such bridges are similar in nature to those seen later at metaphase (see for instance Ris, 1942), but whether they represent viscous connections that persist after a former contact or are indicative of a "reaching out" of chromosomes toward each other, it is impossible to decide.

When the nuclear membrane finally disappears, this picture undergoes marked and sudden changes. The chain of chromosomes, now free of the influence of the membrane, seems to collapse inwardly, frequently forming a closed ring at first and then an irregular aggregate in the middle of the nuclear space. In the many cells seen at this and the following stages no instance of more than a single aggregate has ever been observed, a point of difference with the case of Brachystethus (Schrader, 1946). Concurrently with these changes of orientation there occur alterations in the chromosomal structure. These are marked especially by a partial return to the diffuse condition in the autosomes, with an accentuation of the equational split. The two large autosomes do not seem to become quite as diffuse as the rest, but this difference is not a striking one at best. This return to a more diffuse state causes the autosomal aggregate to appear as a spongy and vacuolated mass in both gentian violet and Feulgen preparations and this condition is maintained for the major part of the first division (Fig. 23). Hematoxylin slides allow no such structural diagnosis for there the aggregate is nearly always homogeneously and intensely stained.

The behavior of the sex chromosomes is remarkable during the prometaphase and the establishment of the metaphase itself. At the time of the equatorial ring formation, just prior to the disintegration of the nuclear membrane, they are still very close or even in contact with the large autosomes. Almost always they lie on the inner side of the ring and not in seriation with the rest of the chromosomes (Fig. 22). They seem to be almost fully condensed at this time and are recognizable in most cells. When the autosomal chain collapses to form the irregular aggregate, this distinction becomes even more marked, for in contrast to the autosomes they then maintain their condensed state and in addition tend to protrude from the spongy mass of autosomes (Fig. 24). This protrusion seems to be an indication of interaction with the two centers, for the sex chromosomes not only make their appearance on the side toward one of the poles but begin to place their long axis in alignment with the polar axis (Fig. 25) which is a placement assumed also at the ensuing metaphase.

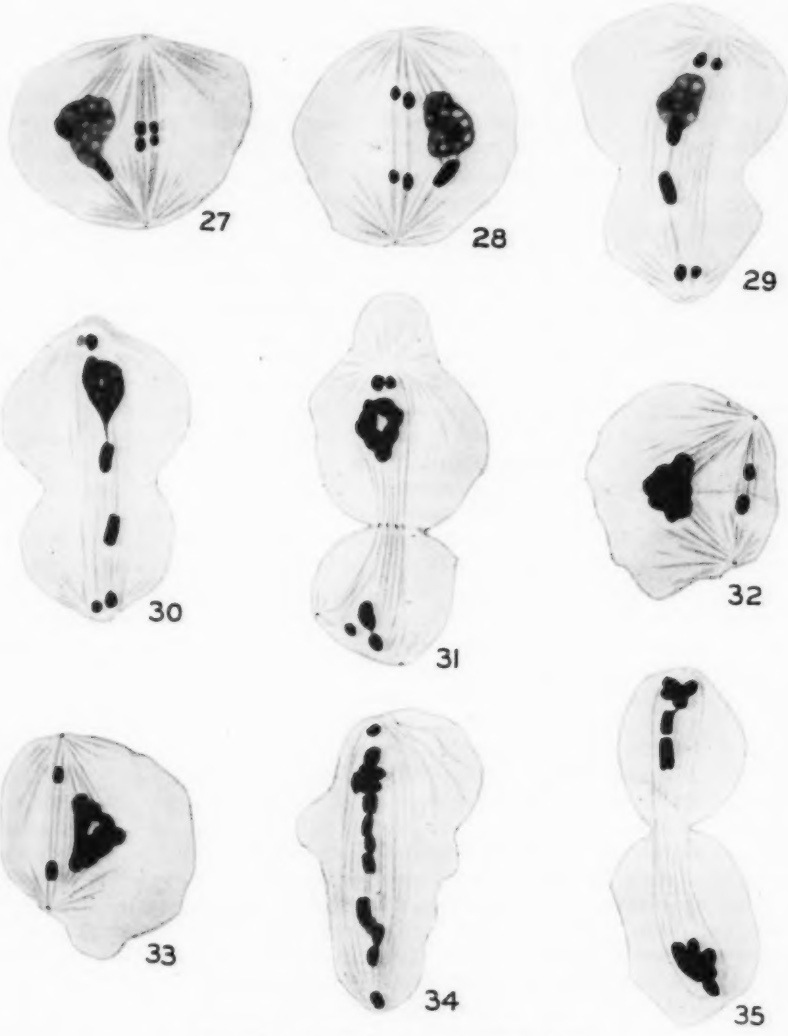
The first division

The clumping of the partially diffused autosomes brings about a rather anomalous situation and the establishment of the metaphase can be followed only through the behavior of the sex chromosomes. These appear to be quite normal in their further maneuvers. They finally become completely detached from the spongy aggregate of autosomes and take up an equatorial position side by side. During this movement several other developments occur simultaneously. Chromosomal fibers appear connecting the sex chromosomes as well as the aggregate with the poles, and at the same time the whole mass of autosomes is shunted out of the middle region toward the side of the cell. This shift must be rather sudden, for intermediate stages are very rare. In extreme instances the displacement may bring the autosomal aggregate very close to the side of the cell, though never touching it, and in every case it comes to lie farther from the polar axis than from the cell wall. While in this position, two points are to be noted: the autosomal aggregate remains connected with the poles through definite chromosomal fibers, and even in its displacement it maintains an equal distance from both poles (Figs. 26, 73, and 74). The whole reaction is obviously closely akin to a similar one observed in the pentatomid Brachystethus (Schrader, 1946).

The two sex chromosomes apparently are not affected by the anomalous behavior of the autosomes. They lie side by side in a compact and narrow spindle of normal length and undergo an orthodox equational division. In some cells the chromatids of the Y separate faster than those of the X and may precede them to the poles. As soon as the anaphase movement of the sex chromosomes is initiated, the autosomal aggregate once more approaches the polar axis, and by mid-anaphase is usually close to or even in contact with the sex chromosome spindle. This return also occurs in the equatorial plane of the cell, and is correlated with a shortening of the chromosomal fibers as well as the lengthening of the interpolar distance and cell as a whole—both of which will of course bring the autosomal aggregate closer to the polar axis again (Figs. 27 to 29).

Although the autosomes, aggregated as they are, pass through these maneuvers as a unit, there is evidence from the beginning that one of them plays a special role. Already at the first trace of division in the X and Y chromosomes, a single large chromosome protrudes from the autosomal clump, showing a well formed chromosomal fiber connection with one center and clearly oriented toward it. In such a position it appears more condensed than the rest of the autosomes, a condition which would be difficult to discern while it is still in the midst of the vacuolated, unevenly staining aggregate (Figs. 27 and 28). The reaction of this autosome to the pole is quite independent of the sex chromosomes, but it is obviously hindered in its movements—probably because of the "stickiness" that tends to hold all the autosomes together. As a result the two sex chromosomes are well on their way toward the poles before this autosome has disengaged itself from the encumbrance (Figs. 29 and 75).

Soon after it has left the aggregate, a second large autosome begins to dissociate itself from the rest of the autosomes. The extent to which it succeeds in this is highly variable in different cells, but in most cases it at least protrudes from the mass before the division is finished (Fig. 30). Often, while the first autosome is still fairly



Mecistorhinus melanoleucus—Harlequin Lobe

FIGURE 27. Early anaphase I; autosomal aggregate beginning to return (Gentian violet).
FIGURE 28. Mid-anaphase I; (Gentian violet).
FIGURE 29. Late anaphase I (Gentian violet).
FIGURE 30. Early telophase I (Gentian violet).
FIGURE 31. Late telophase I (Hematoxylin).
FIGURE 32. Early anaphase II—large cell (Gentian violet).
FIGURE 33. Mid-anaphase II—large cell (Hematoxylin).
FIGURE 34. Early telophase II—large cell (Hematoxylin).
FIGURE 35. Telophase II—large cell (Hematoxylin).

close to the aggregate, it shows a Feulgen-positive connecting thread with the second one, although this is usually severed very shortly (Fig. 29).

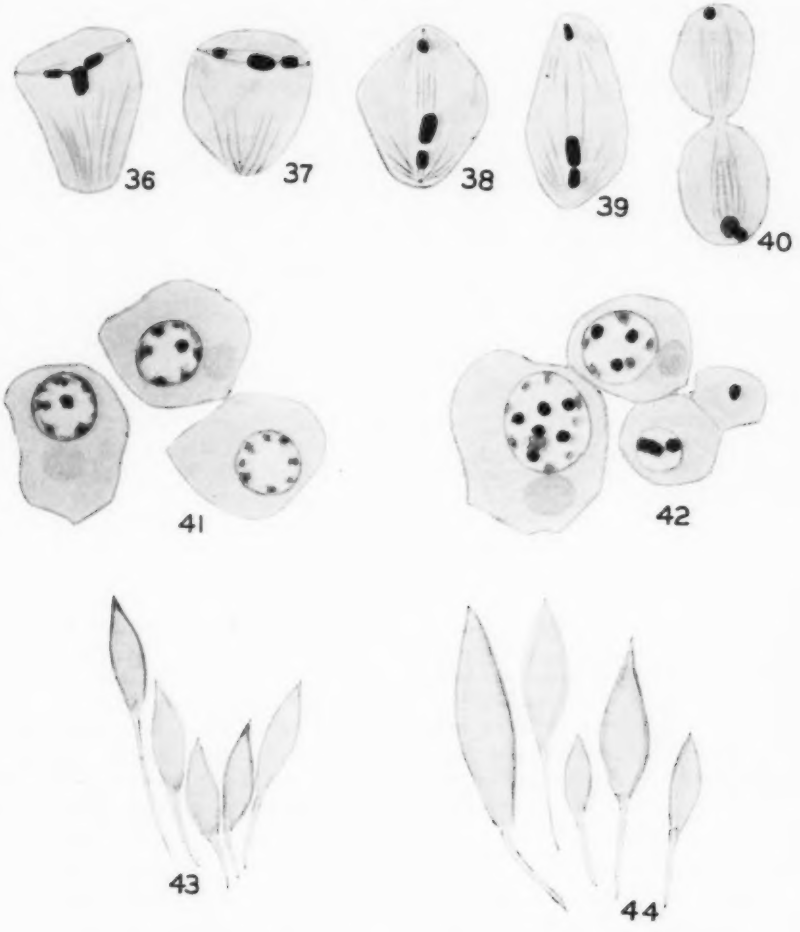
The rest of the autosomal clump, which is by now very close to the polar axis, also shows some response to the mitotic forces and frequently undergoes some elongation in the polar direction. Its effective movement however is always opposite in direction to that of the first autosome and hence it approaches the other pole. The second large autosome may sometimes almost reach the middle of the cell, but in only a single case has it proceeded so far that the ensuing cleavage constriction promises to include it in the same cell with the first autosome. Indeed, a count of 100 small second spermatocytes (those which do not receive the main part of the autosomal aggregate) has revealed no such accidental inclusion and it must be very rare. Further, in all observed cases (of which there are many dozens) this second autosome has rejoined the aggregate by the time that the second division is begun so that its mitotic motion must finally be reversed (Fig. 31). Possibly it initially follows the large autosome only because it is dragged along by the connecting thread. The final result of these maneuvers is that the large autosome and the rest of the autosomal aggregate always go to opposite poles. Since the former is the first to evince any reaction to the mitotic forces, one is almost forced to the hypothesis that it determines the direction of movement on the part of all the remaining autosomes.

These two autosomes that tend to disengage themselves from the aggregate are patently larger than any but the two largest chromosomes of the diploid set. At the same time they do not seem quite to reach the size of that large pair and it may therefore be that we are dealing with the two chromatids of only one of the latter. But admittedly the changes in the state of autosomal condensation during meiosis make it difficult to decide the matter on the basis of size alone. The later behavior of these exceptional chromosomes in the spermatids would however seem to support their identification as chromatids rather than whole chromosomes.

The second spermatocytes resulting from this first division, anomalous though it may be, are thus very constant in composition. Half of them contain the two sex chromosomes and only one autosome; the rest carry all the remaining autosomes as well as the sex chromosomes. The latter are much the larger cells of the two, as might be expected (Fig. 31).

The second division

Large cell: The chromosomes carried by these second spermatocytes comprise the sex chromosomes as well as all of the autosomes except the single one in the smaller cell. There is no interkinesis. In its general aspects the division of this cell simulates the first division. As the X and Y take their position on the new spindle, chromosomal fibers are also formed between the two poles and the autosomal aggregate. Almost simultaneously, the latter is shifted toward the side of the cell, the displacement being very similar to that observed in the first division (Figs. 32 and 76). However the aggregate now begins to be less spongy in appearance and there are other indications that its autosomal constituents are once more undergoing condensation (Fig. 33). During the anaphasic separation of the X and Y, the aggregate is again drawn toward the middle of the cell. It may be noted that when it reaches the compact little sex chromosome spindle, its surface of contact with it becomes smooth and concave, indicating that it is under some pressure (Fig. 33).



Mecistorhinus melanoleucus—Harlequin Lobe (except Figs. 41 and 43)

FIGURE 36. Beginning of Division II—small cell; large autosome connected with both X and Y (Hematoxylin).
FIGURE 37. Early anaphase II—small cell (Hematoxylin).
FIGURE 38. Late anaphase II—small cell (Hematoxylin).
FIGURE 39. Early telophase II—small cell (Hematoxylin).
FIGURE 40. Late telophase II—(Hematoxylin).
FIGURE 41. Early spermatids—normal lobe (Hematoxylin).
FIGURE 42. Early spermatids—harlequin lobe (Hematoxylin).
FIGURE 43. Late spermatids—normal lobe (Hematoxylin).
FIGURE 44. Late spermatids—harlequin lobe (Hematoxylin).

Then as the anaphase progresses and the cell and spindle elongate, the aggregate is drawn out between the two poles and many of its component autosomes are thus strung out in a roughly linear order (Figs. 34 and 78). But the tendency to stick and adhere to each other remains strong so that even when several individual chromosomes have been pulled out of the aggregate they usually still show thick, Feulgen-positive connections with each other and with the remaining aggregate (Figs. 34 and 35). The division so far as the autosomes are concerned is therefore obviously a haphazard one and the aggregate is frequently distributed very unevenly to the two spermatid cells.

The spermatids resulting from the division of the large second spermatocyte are therefore exceedingly variable in composition. Since the sex chromosomes segregate normally, all spermatids carry either an X or a Y, but the number of autosomes is largely a matter of chance. Nevertheless no cases have been observed in which the latter have all gone to one pole.

Small cell: As is the case with the large second spermatocytes there is no interkinesis of any kind and the final stages of the first division merge directly into the beginnings of the second. Indeed before the anaphase movement of the first division has been completed, the two daughter centrioles at each pole (each centriole appears double already at metaphase) have separated and begin their migration to establish the polar axis of the second division, at right angles to the first (Fig. 31). In some cells the sex chromosomes respond and orient to these new poles before they have completed the anaphasic movement. But apparently this precocious movement is then reversed, for in slightly later phases when the new axis has been established, the two sex chromosomes are near each other or in actual contact in the middle of the cell. Here too now lies the large autosome which has been delayed in its arrival at the pole. It may be in contact with neither, either or both sex chromosomes, or it may be connected with either or both through Feulgen-positive bridges (Fig. 36).

Chromosomal fibers are formed already before the three chromosomes have gathered at the midpoint, but so far as can be seen the tiny spindle is concerned only with the X and Y. No chromosomal fibers can with certainty be traced to the autosome. The sex chromosomes behave just as they do in a normal cell and after meeting in the middle of the spindle they separate to opposite poles in a regular segregation.

It is the behavior of the autosome that presents some puzzling aspects, as it already has done in the first division. The outstanding feature of this behavior lies in the fact that it nearly always goes to the same pole with the X. In so doing it appears to have little or no independent mitotic movement, acting merely as a satellite of the sex chromosome. Its dependence on the latter is shown not only by the absence or poor development of chromosomal fibers already mentioned, but is indicated also by its behavior before and during anaphase. In the grouping prior to the division, the autosome sometimes lies between the X and the adjacent pole, but when the anaphase movement is under way it always trails the sex chromosome (Figs. 36 to 40, and 77). Since during most late anaphases the autosome is attached more or less closely to the X, its behavior might be attributed to a simple adhesion between the two were it not for the fact that many earlier anaphases show no such connection. The latter thus frequently seem to be established after the mitotic movement is under way. Indeed, connections of this sort cannot be decisive in any case since prior to the division the autosome often lies in contact with both

sex chromosome or shows a Feulgen-positive bridge to the Y and not to the X (Fig. 36). Evidently this is later broken so that the mitotic association with the X must involve some selective action not dependent on such physical bonds. Whatever the underlying mechanism may be, the results of the division admit of no doubts concerning the constancy of the relationship between these two chromosomes. In 100 clear side views of late anaphases there were only six that admitted the possibility of an association of the autosome with the Y. In two of these the autosome is clearly much closer to the Y than the X and probably going to the same pole, whereas in the remainder the identification of the X and Y is not certain. In close to 95 per cent of the cases therefore, the autosome accompanies the X to the pole and not the Y. Generally speaking then, the small spermatids which come from this division are of two types: one, which carries the X and the autosome; and another which contains only the tiny Y.

Spermatids and sperms

The two anomalous spermatocyte divisions of the harlequin lobe thus give rise to four main types of spermatids: X + one large autosome; Y; X + a variable number of autosomes; Y + a variable number of autosomes. Since in the spermatid the autosomes scatter again and tend to be distributed peripherally on the new nuclear membrane before becoming diffuse, rather dependable counts are often possible. Both of the small types of spermatids are readily recognizable and it is to be noted that the close association between the X and the autosome of the preceding telophase is maintained into an advanced spermatid stage (Fig. 42). In the large spermatids the number of chromosomes may be as low as five or six and frequently higher than twenty. The latter counts constitute conclusive evidence that the chromatids of the univalent autosomes have separated from each other, since the full, normal haploid number of the species is only seven (compare the normal spermatids of Fig. 41 with those of the harlequin lobe in Fig. 42). Since the autosome that is associated with an X in one of the small spermatids undergoes no such separation into smaller units, there is strong presumptive evidence (to support that which was adduced earlier) that it represents a chromatid which has already separated from its sister chromatid—the second large autosome of the first division.

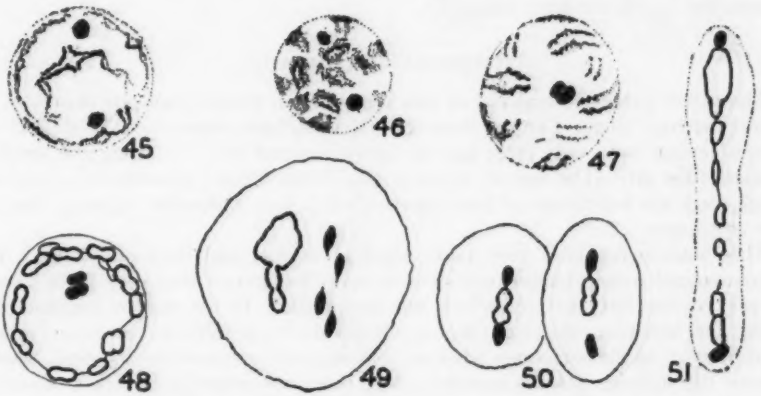
Formation of sperms seems to proceed normally in all cells until the stage when the elongation nucleus shows the pointed apex which indicates the presence of the acrosome (compare Fig. 43 with Fig. 44). At about this time, when the chromosomes have become very diffuse and the contents of the sperm head seem structureless, the small types of sperms gradually stain more and more faintly. It finally becomes impossible to recognize them, whereas the larger types assume the attenuated, intensely stained form of the head that is also encountered in the normal lobes. It is more than probable therefore that the smaller sperms never attain maturity, whereas the large ones appear normal in every respect but size. They enter the sperm duct in a perfectly regular manner and there mingle with the sperms of the normal lobes.

Mecistorhinus tripterus

The main points in which *Mecistorhinus tripterus* differs from *Mecistorhinus melanoleucus* involve the heteropycnosis of the autosomes and the behavior of the

sex chromosomes. So far as the diploid complement of chromosomes is concerned, there is no perceptible difference, both having an outstandingly large pair of autosomes and a Y that is the smallest of all the chromosomes. However, already in the spermatogonial resting phases there is a less extensive heteropycnosis in *Mecistorhinus tripterus,* for then as well as later there are never more than two heteropycnotic bodies (instead of from three to five). The spermatogenesis in the normal lobes is entirely orthodox. The heteropycnosis in the large autosomes is here lacking entirely and hence there is no association between them and the sex chromosomes (Fig. 45).

The lack of autosomal heteropycnosis obtains also in the harlequin lobe (Figs. 46 and 47). As a consequence, when the equatorial ring is formed the two sex



Mecistorhinus tripterus—Harlequin Lobe (except Fig. 45)

FIGURE 45. Normal diakinesis; X and Y independent of autosomes (Feulgen).
FIGURE 46. Early diplotene stage (Feulgen).
FIGURE 47. Diakinesis; X and Y joined (Feulgen).
FIGURE 48. Equatorial ring in formation; X and Y independent of autosomes (Feulgen).
FIGURE 49. Anaphase I (Feulgen).
FIGURE 50. Anaphase II—small cell (Feulgen).
FIGURE 51. Anaphase II—large cell (Feulgen).

chromosomes lie in the approximate middle of the nuclear space instead of being carried to the periphery by the large autosomes (Fig. 48). The X and Y are at this time still in more or less intimate contact with each other and remain so until the first anaphase has begun.

Another difference lies in the size relations between the X and the Y. In *Mecistorhinus melanoleucus* the X is markedly larger than the Y, but this difference is less pronounced in *Mecistorhinus tripterus.* In the latter also, as in most other pentatomids, the sex chromosomes become less sharply outlined in the second division and therefore it then becomes difficult at times to distinguish between them (Fig. 50). As a consequence, in about 25 per cent of fifty cells it is not possible to decide whether the autosome follows the X and there is at least the possibility that in such

instances it accompanies the Y. Although there is thus no question that in this race too the association is between the X and the autosome in the great majority of cells, the case is not as clear-cut as it is in *Mecistorhinus melanoleucus*.

Mention should also be made of the fact that already in the first division, the first large autosome usually shows a median furrow (Fig. 49). If, as in *Mecistorhinus melanoleucus* this chromosome represents only one chromatid of one of the large univalents, the furrow is equivalent to a tertiary split. Whether that be correct or not, this split is not consummated even in the early spermatid, where the large autosome still maintains this appearance.

But in essence, the meiotic divisions of the two species are very much alike (Figs. 49–51, and 78). The difference in heteropycnosis does not affect the results and *Mecistorhinus tripterus* merely furnishes less decisive evidence anent the association between the X and the large autosome.

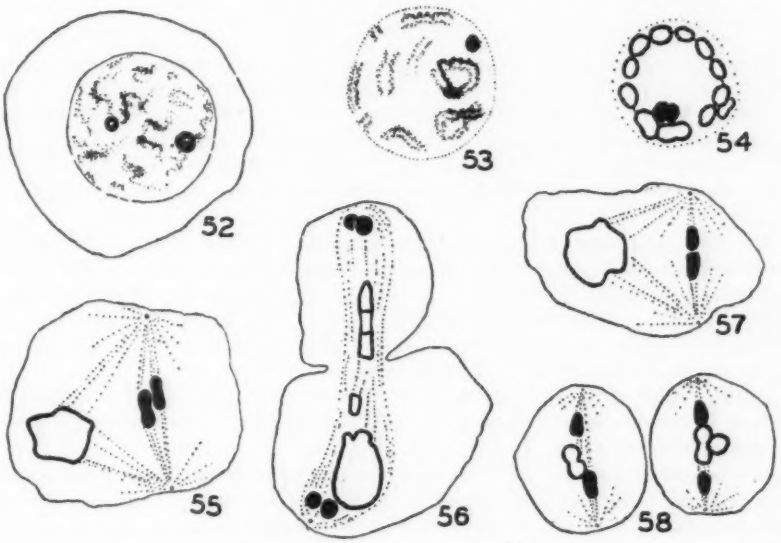
Mecistorhinus sepulcralis

The rather extensive material of this species from Brazil conforms closely in its general cytology to the Central American Mecistorhinus species. The diploid set is characterized by a very large pair of autosomes and the Y is again the smallest member of the set. The meiosis in the normal lobes follows an orthodox course although there are indications of heteropycnosis in a very restricted region of the two large autosomes.

This heteropycnosis is very much alike in normal and harlequin lobes. The spermatogonial resting phases may show as many as three or four very small heteropycnotic regions but more often only one larger one. In the meiotic prophase following the leptotene stage and up to diakinesis, it is difficult to recognize any heteropycnosis in the autosomes whereas both sex chromosomes are, as usual, heteropycnotic throughout. In the confused stage there are generally two heteropycnotic bodies, but whether these represent only the sex chromosomes or also certain autosomal regions it is not possible to say (Fig. 52). Only in mid-diakinesis do conditions allow a closer analysis. In the harlequin lobe, where we are dealing with univalents, both large autosomes then show heteropycnotic terminal regions, and these are joined so as to form closed rings as in *Mecistorhinus melanoleucus* (Fig. 53). Apparently this heteropycnosis is less extensive in the present species and this may account for the fact that the mutual attraction of such regions does not bring the two large univalents together in the "figure eight" formations that occur so often in the other forms. Probably for the same reason the association between the sex chromosomes and these autosomes is highly variable. Thus in some cells only one sex chromosome is joined to the heteropycnotic region of one univalent autosome while the other sex chromosome is free (Fig. 53). In late diakinesis such a condition is rare because, as in *Mecistorhinus tripterus*, there is then a strong tendency for the X and Y to come together. Hence the free sex chromosome often joins its attached mate and as a result both are carried into the equatorial ring of autosomes, just prior to the breakdown of the nuclear membrane (Fig. 54). In other cases the sex chromosomes may be entirely free and will then take a more or less central position in the nucleus at the time of the equatorial orientation of the autosomes.

But these various maneuvers that involve heteropycnotic attraction do not affect the course of the actual divisions in the harlequin lobe any more than in the normal

lobes. In the former the sex chromosomes at both metaphases become detached from the autosomal aggregate, which then is displaced toward the side of the cell just as in *Mecistorhinus tripterus* (Figs. 55 and 57). In *Mecistorhinus sepulcralis* however, the first anaphase shows a definitely greater tendency for other chromosomes to follow the first large autosome out of the aggregate. Although the second large autosome is included as rarely in the smaller second spermatocyte as in the preceding species, a smaller autosome sometimes succeeds in following the first autosome into this cell (in four out of forty cells) (Fig. 56). Apparently both these autosomes



Mecistorhinus sepulcralis—Harlequin Lobe

FIGURE 52. Confused stage (Feulgen).
FIGURE 53. Diakinesis stage (Feulgen).
FIGURE 54. Equatorial ring; X and Y associated with large autosomes (Feulgen).
FIGURE 55. Metaphase I (Hematoxylin).
FIGURE 56. Late anaphase I (Hematoxylin).
FIGURE 57. Metaphase II—large cell (Hematoxylin).
FIGURE 58. Anaphase II—small cell (Hematoxylin).

retain the "sticky" condition of the earlier aggregate, for when they reach the pole the smaller joins the larger one and adheres to it in almost any position in random fashion. In the second division, both follow the X to the pole in the majority of cases (Fig. 58).

But here too, as in the second division of *Mecistorhinus tripterus* the X and Y often show only a small size difference In perfect side views they can nearly always be distinguished, but if the cell is viewed at a slight angle it at once becomes difficult to do so. Hence, though *Mecistorhinus sepulcralis* also shows a definite preferential association between the X and the large autosome (as well as the smaller, if present)

the possible occurrence of exceptions to the rule can no more be excluded than in the preceding form.

The division of the aggregate in the larger second spermatocyte occurs very much as it does in *Mecistorhinus tripterus*. The four main types of spermatids are therefore alike in all three species, comprising X + one autosome; Y; X + variable number of autosomes; Y + variable number of autosomes. The few exceptions in the present species are due to the occasional inclusion of a smaller autosome in the first named spermatid.

Neodine macraspis

This representative of another genus is in its cytological features almost a duplicate of *Mecistorhinus tripterus*. The cells and chromosomes of the harlequin lobe are slightly larger than those of the preceding forms. The large pair of autosomes however does not stand out quite so strikingly in its size as in the species of Mecistorhinus (Fig. 59). The normal meiosis is entirely orthodox (Figs. 60 to 63) and there is no heteropycnosis other than that of the sex chromosomes to complicate the prophases.

The harlequin lobe likewise lacks all autosomal heteropycnosis. The two sex chromosomes come into contact with each other during the confused period and this loose union is maintained through the diakinesis (Figs. 64 to 66) into the first metaphase (Fig. 67). They are entirely independent of the autosomes, and when the latter are marshalled to form their equatorial ring the X and Y are always found together in the middle of the nucleus (Fig. 66).

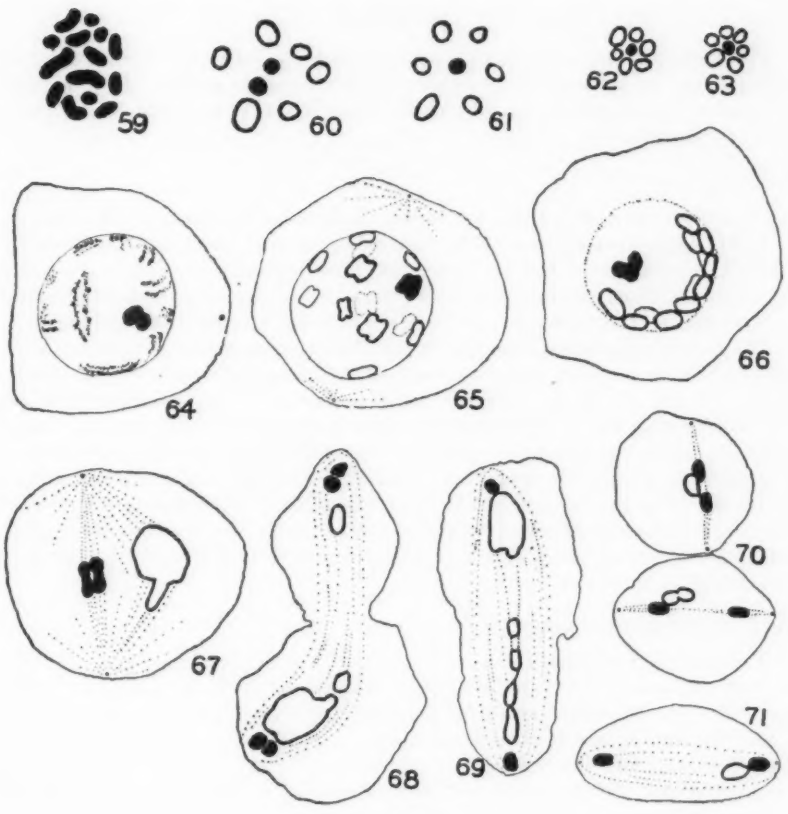
In the course of the first division, only one large autosome and no others join the X in the formation of the smaller second spermatocyte. This autosome may or may not exhibit the median tertiary split (Fig. 70).

The second division conforms to the general scheme described for the other species. The size difference between the X and Y is not as clear cut as in *Mecistorhinus melanoleucus*, but there is no doubt that in the great majority of cells, it is the X with which the single autosome is associated and not the Y (Figs. 70 and 71). The larger second spermatocyte divides irregularly and in the same manner as does the corresponding cell in Mecistorhinus.

In all essentials, therefore the meiosis in the harlequin lobe of Neodine parallels that of Mecistorhinus.

Platycarenus notulatus

In view of the uniformity in the occurrence of normal and harlequin lobes of the four preceding species, it comes somewhat as a surprise that in another species, *Platycarenus notulatus*, the conditions are entirely different. The structure of the testis departs radically from that of the other forms, there being only four instead of seven lobes, with a more compact instead of a serial arrangement. The diploid set of fourteen chromosomes is marked by no strikingly large pair of autosomes and only the exceptionally small size of the Y merits any notice. There is no harlequin lobe and the spermatogenesis takes a very orthodox course throughout the testis. The case thus serves warning that the cytological conditions are by no means uniform in the tribe Discocephalini as it is now constituted by the systematists.



Neodine macraspis

Normal Lobe:

FIGURE 59. Spermatogonial metaphase (Hematoxylin).
FIGURE 60. Metaphase I (Hematoxylin).
FIGURE 61. Metaphase II (Hematoxylin).
FIGURE 62. Telophase II; polar view with Y (Hematoxylin).
FIGURE 63. Telophase II; polar view with X (Hematoxylin).

Harlequin Lobe:

FIGURE 64. Early diakinesis; joined X and Y independent of autosomes (Hematoxylin).
FIGURE 65. Late diakinesis; 12 univalents + joined XY (Hematoxylin).
FIGURE 66. Formation of aggregate; joined X and Y separate (Hematoxylin).
FIGURE 67. Early anaphase I (Hematoxylin).
FIGURE 68. Telophase I (Hematoxylin).
FIGURE 69. Telophase II—large cell (Hematoxylin).
FIGURE 70. Anaphase II—small cell (Hematoxylin).
FIGURE 71. Telophase II—small cell (Hematoxylin).

Discussion

The most striking anomalies of the meiosis in the harlequin lobe of the Discocephalini may be summed up as follows: 1) Omission of pairing, 2) Clumping or aggregation of autosomes, 3) Preferential association between the X chromosome and a certain autosome.

Omission of pairing

The failure of the leptotene homologues to come together in synapsis has at present no cytological explanation. The only indication that conditions are not entirely orthodox at an earlier stage lies in the fact that the leptotene threads are less fine and attenuated than they are in normal lobes. In short they are not despiralized to the same extent as chromosomes that undergo the characteristic pairing reaction.

The further prophase behavior of the unpaired chromosomes is however of great interest. These univalents pass through all the meiotic phases that distinguish bivalents from the chromosomes of non-meiotic mitoses. They rapidly condense after the leptotene stage and a diplotene split makes its appearance; there follows a regular confused period; and the latter is in turn succeeded by an entirely typical series of diakinetic maneuvers. It would seem therefore that these characteristic prophase manifestations of meiosis are not at all conditioned by the phenomenon of pairing and, given certain basic conditions, the chromosomes run through the gamut of meiotic changes even when this most obvious feature of the meiotic prophase has been omitted.

Clumping and displacement of autosomes

The clumping or aggregation of autosomes which occurs suddenly just before the first metaphase and is not completely abandoned until the spermatid stage, is one of the most striking features of the harlequin lobe. It is only natural to seek some interrelation between this anomaly and the failure of synapsis that precedes it, but the connection is certainly not a direct one. Thus the omission of pairing in Loxa (Schrader, 1945b) is followed by no clumping in the first division (although there are indications of "stickiness" in the second). The most decisive evidence against such an interrelation is furnished by Brachystethus (Schrader, 1946). There the clumping of autosomes is even more persistent than in the Discocephalini but an omission of pairing obviously cannot be held responsible since the autosomes undergo normal synapsis and form typical tetrads.

The occurrence of autosomal heteropycnosis in the prophase of *Mecistorhinus melanoleucus* can also not be held responsible for the later aggregation of autosomes. In *Mecistorhinus tripterus* the clumping is just as marked as in the first named species, but there is no heteropycnosis in the autosomes at all. Indeed, despite the peculiar maneuvers that such heteropycnosis brings about especially during diakinesis, the meiotic divisions themselves are not perceptibly affected. They are essentially the same, whether autosomal heteropycnosis be present or not.

In all these considerations of the clumping phenomenon it must be remembered that the mitotic apparatus remains normal and cannot be held directly responsible for the anomalies. The justification for this conclusion lies not only in the fact that chromosomal spindle fibers are regularly formed between the centrioles and the

clumped autosomes, but also in the perfectly normal spindle relations of the two sex chromosomes.

It is the orthodox behavior of the sex chromosomes that may furnish a clue to the autosomal behavior. As usual, both the X and the Y chromosome are persistently heteropycnotic and at first metaphase are fully condensed. The autosomes however depart from their usual cycle of condensation and diffuseness. Already at prometaphase their precocious reaction to the polar forces would suggest a condition at variance with that which normally obtains—perhaps a more advanced state of condensation. Such abnormal timing is indicated more directly when the disintegration of the nuclear membrane finally occurs. The autosomes have then begun to reverse the normal sequence of development and when they undergo the clumping reaction they are in process of returning to the diffuse condition rather than attaining their final condensation. It is this untimely regression in nucleination (for there appears to be only a slight uncoiling) that probably underlies their clumping.

Such a hypothesis implies a close correlation between the state of condensation and the mutual reactions of chromosomes. It may be objected that during a normal prophase, when the chromosomes pass through all stages of condensation, no such clumping ever occurs. But it must be remembered that during the entire prophase a nuclear membrane is present and there can now be little doubt that this exerts the most far-reaching influence on chromosomal behavior (Schrader, 1941). Indeed when the membrane finally breaks down there is in normal cells also an immediate clumping of chromosomes which do not separate again until they reach final condensation shortly thereafter. And in the normal telophase, when the chromosomes have started to return to the diffuse condition, there is again a tendency to clump. It is only after the new nuclear membrane has been formed that the chromosomes separate and scatter once more. The so-called mutual repulsion of the chromosomes in these Pentatomidae and perhaps other forms as well thus appears to occur only when they are fully condensed or else are contained within a nuclear membrane.

It is possible that here also lies the key to the extrusion of the autosomal aggregate from the midregion of the spindle. As has already been said, the orthodox behavior of the sex chromosomes in the same spindle proves that it is not in the spindle apparatus that an explanation is to be found. It is the autosomes and kinetochores that must be held responsible and the abnormality in their condition which results in clumping is probably also involved in their lateral displacement. That this aberrant condition is only temporary is indicated by the fact that there is a partial return to normal behavior in the second division. The aggregate of autosomes is then stretched between the two poles and, though still "sticky," many of its components become dissociated from each under the strain and again appear as individual chromosomes. By the time the spermatid stage has been reached their behavior appears to be normal and they scatter over the periphery of the nuclear membrane quite like the chromosomes of the neighboring lobes.

It is safe to assume that the establishment of the metaphase involves a set of precisely adjusted interactions between centers, chromosomes and kinetochores. That these interactions involve nothing more than a system of repulsions would seem to be very unlikely, and indeed the retention of the chromosomes within the compass of the equatorial plate almost demands forces of a positive nature. Whether these be contractile chromosomal spindle fibers or a zone of actual attraction in the equator or both, it is quite possible that an upset in the condition of one of the elements

involved might allow the forces of repulsion to gain the ascendancy.[2] On such a basis the regressive nucleination that is perhaps accompanied by a partial weakening of the kinetochores may well account for the expulsion of the autosomes in these Discocephalini.

The preferential segregation of one autosome

The special behavior of one of the autosomes and its association with the X chromosome has already been described. In analyzing its movements the following factors should be considered:

In the first division this chromosome shows no connection with either of the two sex chromosomes and its mitotic reactions are directed solely to the nearest pole. Its only distinction from the rest of the autosomes lies in a slightly greater condensation which is not very pronounced but may explain why it behaves more normally than they in this division. In the division of the smaller second spermatocyte this independence of the autosome is lost and to all intents and purposes it then becomes a satellite of the X chromosome.

The available evidence suggests that this chromosome represents a single chromatid of one of the large, univalent autosomes. It is probable that the two chromatids of this large univalent separate in a normal mitotic movement and that position in the aggregate determines which one frees itself and joins the two sex chromosomes at one pole. Its mate always moves to the opposite pole and with it goes the rest of the aggregate. The complications that attend these maneuvers arise primarily from the stickiness that tends to hold all autosomes together.

This provisional explanation of the peculiarities of the first division does not however throw much light on the division of the smaller second spermatocyte. The preferential segregation of the autosome is obviously a consequence of some interrelation with the X chromosome. To be sure, the two chromosomes are often connected by a Feulgen-positive strand or direct adhesion to each other. But before anaphase such a connection is sometimes also established between the autosome and the Y, and in still other cases there is no visible connection with either sex chromosome. Nevertheless, in the great majority of cases (about 95 per cent in *Mecistorhinus melanoleucus*) it is the X with which the autosome is finally associated at telophase and not the Y. The evidence as it stands therefore admits of only one interpretation which has only a few parallels in the literature—the preferential segregation of the autosome is due to some kind of attraction between it and the X (or, much more unlikely, its repulsion by the Y). A directed movement is involved in any case and since the X behaves just as it would in a perfectly normal cell it is the autosome itself which is primarily accountable.

Evolutionary aspects

It is a matter of surprise that so striking a feature as is comprised by the harlequin lobe should have escaped the notice of cytologists for so many years. To be sure it has so far been encountered only in the hemipteran family of Pentatomidae, but that particular group of insects has been subjected to more intense cytological study than almost any other comparable group except the orthopteran family of

[2] In a short article that has just come to hand, Östergren (Bot. Notiser, 1945) suggests that if the spindle is a tactoid such an interplay of forces may well be involved.

Acrididae. Work on some 70 species has been published and among the investigators have been such outstanding cytologists as Montgomery, Wilson, and Geitler. To now discover harlequin lobes in some eight genera and eleven species, widely scattered through the family, can hardly mean that their occurrence has simply been overlooked hitherto. We must seek an explanation elsewhere and in such a quest one cannot fail to be struck by one aspect of the situation. It so happens that despite the great number of species investigated all cytological work has been confined to Pentatomidae from the Palaearctic and Nearctic regions. The rich pentatomid fauna of the Oriental, Australasian, and Ethiopian regions has never been touched by cytologists, and only recently have Neotropical species been investigated. It is among the latter that I have encountered all the species that carry harlequin lobes and the question might well be asked whether the occurrence is not correlated with a tropical habitat. But that is not to say that all tropical Pentatomidae are characterized by such lobes for my own investigations have shown that a number of species of such genera as Alcaeorrhynchus, Arocera, Edessa, etc., do not possess it. Nevertheless the correlation is sufficiently striking to make an inquiry into the effects of various factors in tropical conditions on the development of reproductive organs well worth while.

The occurrence of harlequin lobes in four different species of the tribe Discocephalini collected in localities as far apart as Costa Rica and southern Brazil (about 5400 km. or 3350 miles) would seem to justify the conclusion that such a feature was present in the ancestral species of the tribe. Its absence in a fifth, a species of Platycarenus, might be attributable to loss in the course of evolution. But harlequin lobes are found also in some members of entirely different tribes such as the Halyini (unpublished) and the Pentatomini, and in such a remote form as Brachystethus (Schrader, 1946) its cytological character is obviously very similar to that of the Discocephalini. On the basis of our original reasoning we would thus arrive at the conclusion that a harlequin lobe characterized not only the first discocephalinid but was present already in the more remote ancestor of the whole family of Pentatomidae. Its sporadic occurrence at the present time may therefore be representative of evolutionary survivals that are possible only under certain conditions, though of course it is possible that the genetic basis for the harlequin lobe has existed for a long time but that this can express itself only under certain rather limited conditions. In either case, the deciding factor may well lie in the environment which happens to be more often favorable in tropical than in temperate regions.

Since the sperms of the harlequin lobe are ordinarily nonfunctional in the hereditary sense, it is obvious that in such species there is a very great loss of gametes. Natural selection might confidently be expected to eliminate so wasteful a development unless it also confers certain advantages that compensate for the loss of functional sperms. It has been suggested (Schrader, 1946) that these advantages may lie in the nucleoproteins that are carried into the eggs by supernumerary sperms. Polyspermy is of extremely common occurrence in the fertilization of insect eggs and it is probable that the nucleoproteins introduced by the extra sperms are utilized by the growing embryo. The sperms from the harlequin lobe are usually much larger than normal sperms and hence would contribute proportionately larger amounts of nuceloproteins. Species with harlequin lobes may therefore hold an advantage over those that lack them—an advantage that takes the form of an increase in materials that are of special value for the developing embryo. Certain it is that so

prosperous a species as *Mecistorhinus tripterus* which has a distribution from Vera Cruz, Mexico, to São Paulo, Brazil, has found the possession of a harlequin lobe no disadvantage.

The sporadic and yet widespread occurrence of harlequin lobes among the species of Pentatomidae argues strongly that we are dealing with a basic condition. The problem of why a certain lobe in the testis always takes a special course in its development may in the end be no different from that involved in such a case as the development of diverse types of digestive glands from the embryonic gut. In other words, we are confronted here, as well as there, by the old question of differentiation.

Summary

1. In the testes of four species of the pentatomid tribe Discocephalini, the fifth lobe always shows a special type of meiosis.

2. There is no pairing in this lobe.

3. Just prior to metaphase I all the autosomes aggregate in one clump. The sex chromosomes behave quite normally through both meiotic divisions.

4. In the first division the X and Y divide equationally as in normal cells. One large autosome becomes dissociated from the aggregate and reaches the pole opposite to that attained by the rest of the aggregate.

5. A large and a small second spermatocyte result from this first division. In the small one, the single autosome goes to the same pole as the X in the great majority of second divisions.

6. In the division of the larger second spermatocyte, the aggregate is irregularly pulled into two groups.

7. Four main types of spermatids result: X + variable number of autosomes; Y + variable number of autosomes; X + one large autosome; Y. The last two types probably never reach the mature sperm stage.

8. It is pointed out that so firmly established a feature as this seemingly wasteful development in the fith or "harlequin" lobe must have some compensatory advantage, and its cytological and evolutionary significance are discussed.

All figures are photographs from harlequin lobe; Figs. 72 to 77 inclusive from *Mecistorhinus melanoleucus;* Fig. 78 from *Mecistorhinus tripterus.*

Figure 72. Formation of equatorial ring (Hematoxylin).

Figure 73. Early metaphase I with the two sex chromosomes close together in the middle and the autosomal aggregate displaced to one side (Hematoxylin). Shown in two of the cells.

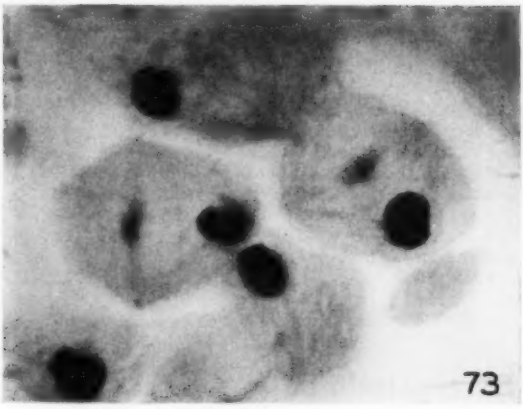
Figure 74. Beginning of anaphase I; X and Y in middle, large autosome beginning to protrude from displaced aggregate (Gentian violet).

Figure 75. Late anaphase I, photographed at two different levels; large autosome leaving aggregate, and an X and Y approaching each pole (Gentian violet).

Figure 76. Anaphase II (large cell); aggregate displaced to one side and X and Y separating to opposite poles (X to lower pole) (Hematoxylin).

Figure 77. Anaphase II (small cell); X with autosome going to upper pole and Y to lower pole (Hematoxylin).

Figure 78. Telophase II (large cell); aggregate becoming dissociated between separating X (lower pole) and Y (upper pole) (Feulgen).



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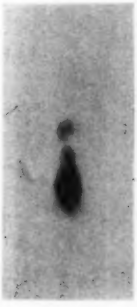

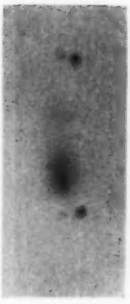

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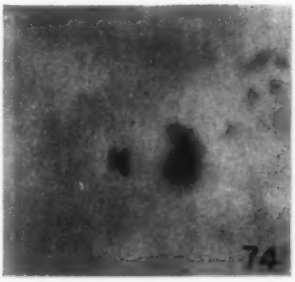

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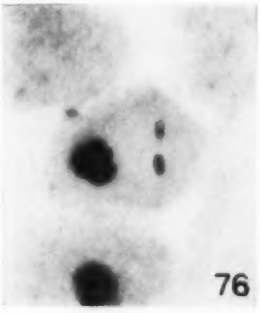

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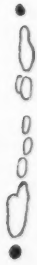

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THE EFFECT OF THE ADULT ANTERIOR PITUITARY HORMONE ON THE TADPOLES AND THE IMMATURE MALE FROGS OF THE BULLFROG, RANA CATESBIANA

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Introduction

The interrelationship of the anterior pituitary hormone and the gonads of vertebrates has been well established. There is evidence that the amphibian gonads, under the influence of the anterior pituitary hormone stimulation, will either respond immediately (in mature animals) by the liberation of mature gametes or will differentiate (if immature) in much the same manner as they do during the breeding season, with the exception that these artificially stimulated changes are accomplished at a much faster rate. This statement is supported by the work of Riddle and Flemion (1928) on the dove; Houssay and Lascano-Gonzalez (1929) on the South American toad; Herre and Raiviel (1939) on larvae of Triton; Burns (1930) and Burns and Buyse (1934) on Amblystoma; Evans (1935) on the lizard; Forbes (1937) on the alligator; Rugh (1937, 1939, and 1941) on frogs of different genera; Puckett (1939) on the bullfrog tadpole, and Glass and Rugh (1944) on the mature frog, *Rana pipiens*. In this latter paper it was shown that while there are seasonal changes in the frog testis, indicating cyclical maturation, the gonad will respond at any time of the year to frog anterior pituitary hormone stimulation by the liberation of any mature spermatozoa which are present, and that frequently (especially during active maturation of August) the seminiferous tubules will also contain other maturation stages. Recently Schreiber and Rugh (1945) have studied the response of the testes of recently metamorphosed *Rana pipiens* to the pituitary hormone and to estradiol-benzoate.

It was the purpose of this investigation to determine the effect of the anterior pituitary hormone of the adult frog on the gonad of the pre- and the post-metamorphosing male bullfrog. The testis can be differentiated from the ovary macroscopically even before tail absorption in the tadpole of *Rana catesbiana*.

Materials and Methods

The tadpoles of *Rana catesbiana* were collected in the vicinity of Woods Hole, Mass., and were used immediately. During the month of August all stages of pre- and post-metamorphosis are available in great abundance. These tadpoles are in their second year of growth, and generally go through the stages of metamorphosis rather rapidly, depending upon the water temperature and the amount of food available. Since these tadpoles were well fed in their natural habitat, there was no attempt to feed them during the short period of the experiment, three to four days.

Tadpoles were selected in five stages:

a. Pre-metamorphic stage where there were four well-developed appendages and a full-length tail fin.
b. Post-metamorphic stage where the body length was less than 45 mm. and the tail had just been absorbed, but could be identified as a stub. These frogs had just shifted to lung breathing.
c. Frogs of body length of 65 mm.
d. Frogs of body length of 95 mm.
e. Mature, full-grown frogs whose body length was at least 120 mm.

These five stages represented a graded series from pre-metamorphosis to the sexually mature adult male, covering a period of normal development ranging from August of one year to the summer of the following year, at least.

The experimental animals were injected within a few hours or, at the most, within 24 hours of collection in the ponds. In most cases the right testis was removed and fixed and the animal was allowed to recover from the gonadectomy and the abdominal wound was permitted to heal for 24 hours. Then each animal was injected with two whole anterior pituitary glands from sexually mature, pre-ovulating female bullfrogs. This is a dose known to be adequate to induce sexual activity in adults (Rugh, 1943). The hormone was injected whole, through a large bore hypodermic needle, into the body cavity, along with about 2 cc. of distilled water. The animal was then placed in a battery jar with a small amount of water and kept at laboratory temperatures. Forty-eight hours later each animal was sacrificed and the remaining (left) testis was removed and fixed in a manner identical with that for the right testis previously removed. In all instances the gonad was removed without direct handling for it has been shown (Rugh, 1939; 1941) that mere manipulation of the living testis will often alter the internal picture. The fat bodies were used as "handles" in excision and removal of the testis. The whole organ was placed in Bouin's solution for 24 hours; slowly run up through to 70 per cent alcohol where the yellow picric stain was removed by the addition of 5 per cent by volume of concentrated NH_4OH for several half-hour changes; dehydration was completed in higher alcohols; clearing in xylol; embedding in a mixture of 90 per cent paraffin (58° MP), 5 per cent bayberry wax, and 5 per cent beeswax. The gonads were sectioned at 8 microns and were stained with Heidenhain's iron-haematoxylin with counterstain.

The photographs were taken on Microcopy film (35 mm.) by means of a Mifilmca adapter.

Observations and Experimental Data

Pre-metamorphic stage

The testis of the pre-metamorphic bullfrog tadpole, which stage possesses all four appendages and a tail, appears as a solid yellowish organ associated with the adjacent reddish kidney by means of the double-layered mesorchium. In section (Figs. 1 and 9) the gonad does not show well-formed seminiferous tubules partly because there is an abundance of interstitial tissue and there are no late maturation stages and no lumina. Scattered around the periphery of the developing seminiferous tubules are found huge primary spermatocytes which seem to be adherent to the base-

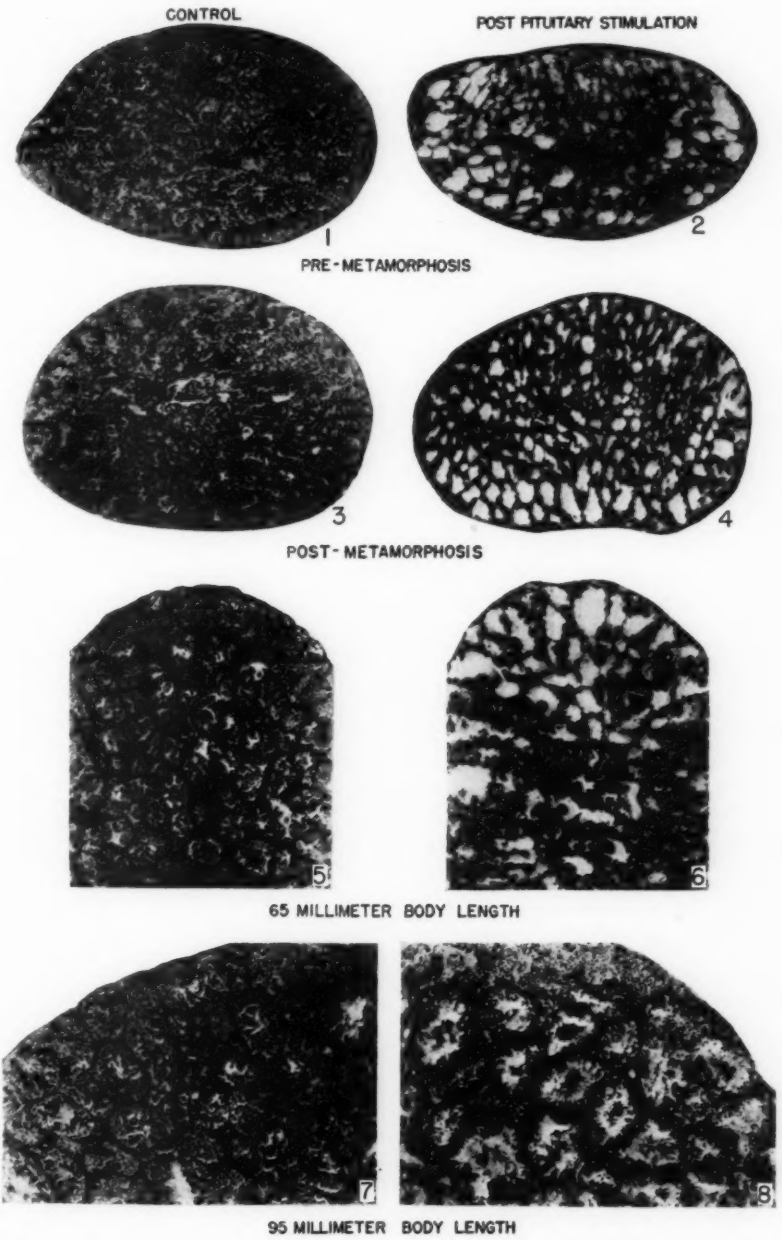


PLATE I. Effect of anterior pituitary hormone on testis of immature bullfrog, *R. catesbiana*.

ment membrane of the tubule. There are also oval-shaped darkly staining nuclei of the spermatogonia, and occasionally there may be seen (toward the region of the future lumen) some secondary spermatocytes. The average length of these testes is 2.18 mm. and the average diameter is 0.80 mm.

When the pre-metamorphic tadpole is subjected to the anterior pituitary hormone (by coelomic injection) the most obvious change in the testis is the vacuolization of the forming seminiferous tubules (Figs. 2 and 10) so that the entire gonad takes on a sponge-like appearance. The peripheral region of the gonad is always the first to be affected, and the most vacuolated. Under high power magnification it is noted that the only cells remaining within the seminiferous tubules are those attached to or an integral part of the basement membrane. The other cells which had previously occluded the lumen will be seen in the collecting tubules and the vasa efferentia, indicating that they were enroute through the reproductive tract at the time the gonad was fixed. One gets the impression that the gonad was flushed out of all loosely attached cellular components.

The size variations of the control and the experimental gonads were not appreciably different.

Post-metamorphic stage, body length less than 45 mm.

In this stage the gonad itself has enlarged considerably so that the maximum length of the testis is about 2.6 mm. and the maximum diameter is about 1.8 mm. The shape of the gonad is also changed, since its length increased about 40 per cent while its diameter increased about 125 per cent.

In the control testis (Figs. 3 and 11) there is some evidence of the forming seminiferous tubules, and maturation stages from spermatogonia to mature spermatozoa are seen. This indicates that coincidental with the drastic changes of metamorphosis the bullfrog tadpole's testis first acquires structurally mature spermatozoa. Whether or not these spermatozoa are functional has not yet been determined. Clusters of cells are seen, all in the same stage of maturation, with the progressively advanced stages more nearly in the center of the seminiferous tubules.

Here again, when the testis is subjected to the anterior pituitary hormone of the adult, there is a liberation from the tubules of all freely associated cells (Figs. 4 and 12) so that the post-stimulated gonad has much the same appearance as that of the experimental testes of the pre-metamorphic stage (Figs. 2 and 10). There remain only the spermatogonia, primary spermatocytes, and the few interstitial cells. The majority of cells found within the collecting tubules and the vasa efferentia (Fig. 17) are secondary spermatocytes.

65 mm. body length stage

By this stage of development the testis has acquired its adult proportions and measures about 3.35 mm. in length and 2.0 mm. in diameter. The surface has become lobulated due to the further development of the seminiferous tubules about the periphery. Nests of maturing cells are seen (Figs. 5 and 13) representing all stages from the primary spermatocytes to the spermatids and mature spermatozoa. The early maturation figures are in great abundance with the pachytene groups the most prominent (Fig. 13). It is at this stage that the cysts or nesting groups of cells become distinct from each other, demarking those clusters of cells, all of which originate from the same spermatogonium. With the enlargement of the seminiferous

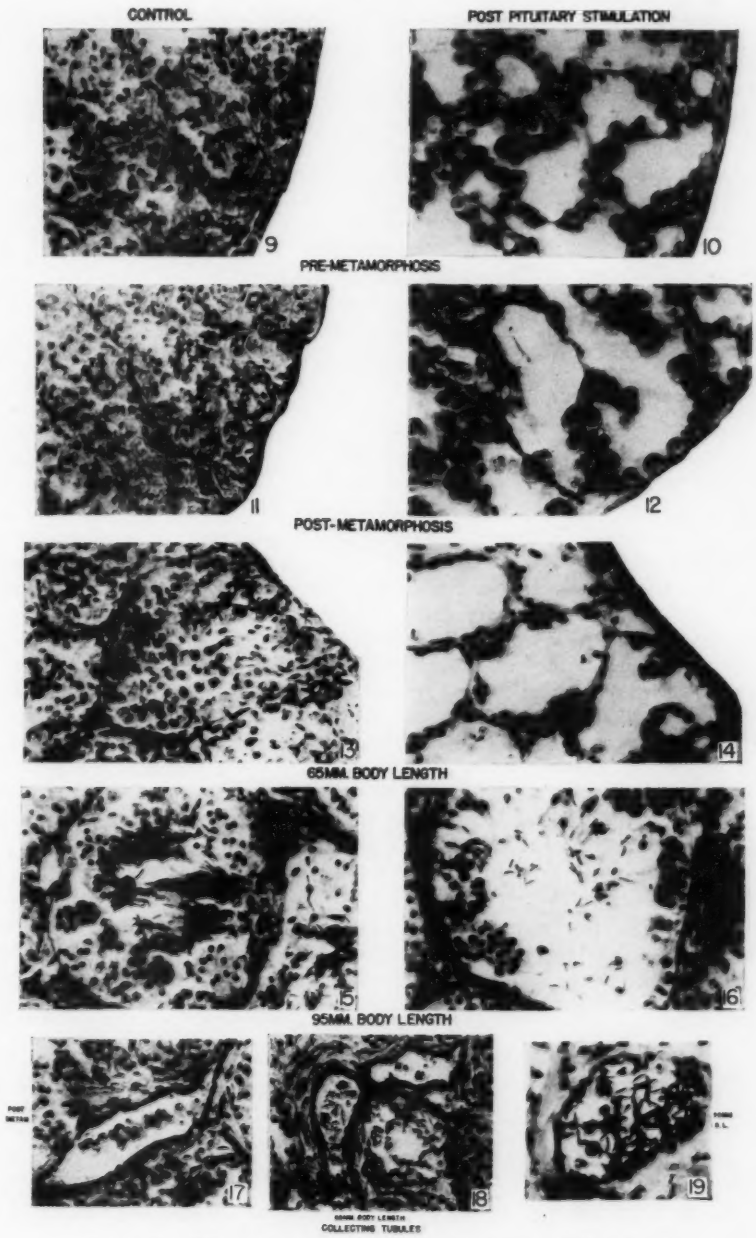


PLATE II. Effect of anterior pituitary hormone on seminiferous and collecting tubules *R. catesbiana.*

tubule there appears to be a compensatory reduction in the amount of interstitial tissue, due, no doubt, to the stretching of this tissue in the general growth of the gonad.

Upon stimulation of this testis by the anterior pituitary hormone of the same species, there is a loss from the gonad of all or most of its freely associated cells and cell groups (Figs. 6 and 14). The gonad seems to have its peripheral tubules emptied the more completely, and, upon closer examination, it is noted that often there remain only the scattered primary spermatocytes and spermatogonia. The primary spermatocytes are fewer in relative number than in the previous stage studied. This is probably due to the fact that some of these cells have been utilized in the production of the maturation nests of cells, some of which were liberated under hormone stimulation. The maturation stages which are missing from the seminiferous tubules are found within the collecting tubules (Fig. 18) and the vasa efferentia leading from the testis to the kidney.

95 mm. body length stage

Except for size, this testis has the histological appearance of the adult gonad. The length is about 5.1 mm. while the diameter is about 2.5 mm. The seminiferous tubules are fully formed and distinct from each other, and the tunica albuginea has become considerably thickened.

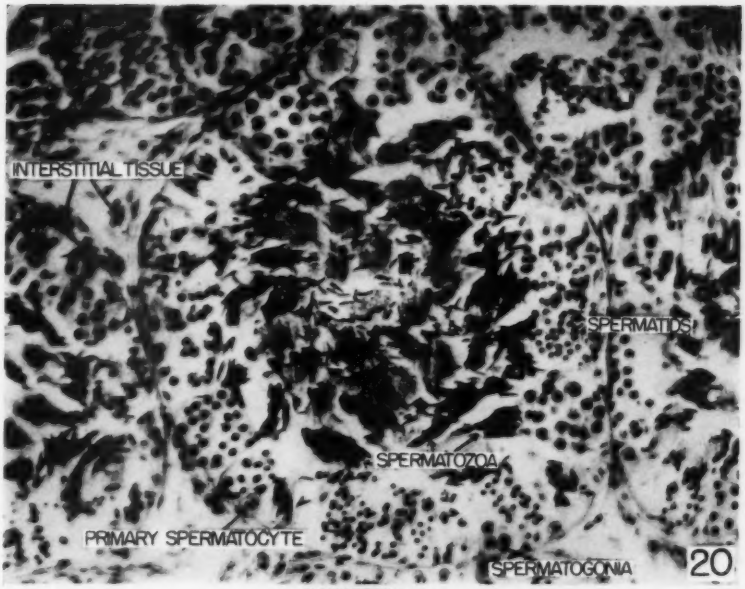
The cysts or nests of cells in the seminiferous tubule include the majority of cells in the more advanced stages of maturation, with abundant fully formed spermatozoa (Figs. 7 and 15). There are many nests of spermatids, and of secondary spermatocytes, but it is with some difficulty that one locates primary spermatocytes.

As a result of anterior pituitary hormone stimulation the lumina of this testis are always enlarged but there is not as complete evacuation of the various cell types as in the previous stage of development studied. That is, some of the secondary spermatocytes and spermatids remain unaffected by the hormone (Figs. 8 and 16), but these same cell types are found within the collecting tubules (Fig. 19) and in the related vasa efferentia.

While the obvious response of the testis as a whole is similar to that of earlier stages of development, there seems to be a greater resistance of many of the cell types, particularly the earlier stages, to evacuation.

The mature bullfrog, body length of more than 120 mm.

The testis of the mature bullfrog measures about 9.8 mm. in length and 5.4 mm. in diameter, being oval in shape and yellowish-white in color. The interstitial tissue is stretched until it remains only as a thin line between seminiferous tubules. Depending upon the season of the year the maturation picture will vary but at the time of this experiment (August) there is normally heightened sexual activity which means abundant mature spermatozoa and abundant reserve cells ready to proceed through maturation, immediately following amplexus and elimination of the mature gametes. Such a picture is seen in Figure 20. It is difficult to locate any spermatogonia or primary spermatocytes, although they are present, due to the abundance of later maturation stages. The mature spermatozoa are in clusters with their heads buried in the cytoplasm of Sertoli cells, and with their tails filling the lumina of the seminiferous tubules. Such a testis is excellent for the study of all of the



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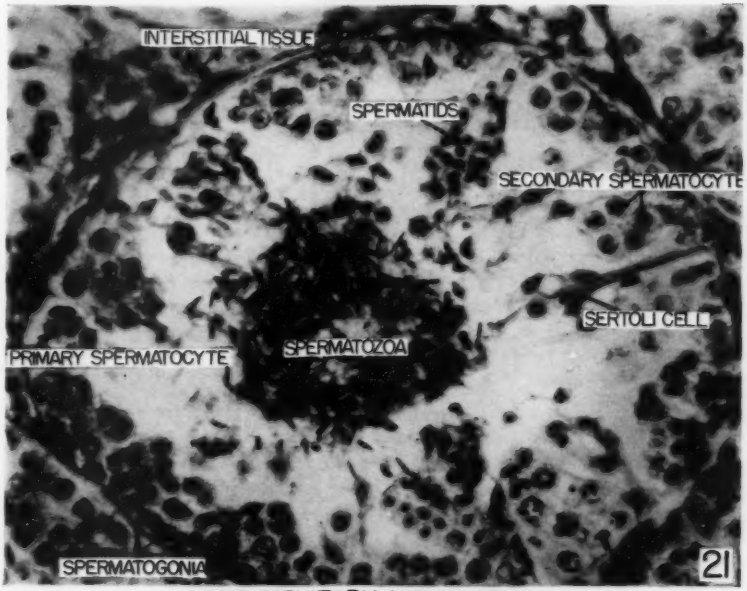

POST PITUITARY STIMULATION

PLATE III. Seminiferous tubules of adult *Rana catesbiana*.

steps in maturation, but, in contrast with the younger frogs there are relatively few of the early maturation stages, i.e., spermatogonia.

Upon stimulation by anterior pituitary hormone the picture becomes somewhat clearer, for the seminiferous tubules are emptied of the mature spermatozoa and an occasional spermatid, and consequently the other stages of maturation within the seminiferous tubules become visible (Fig. 21). The collecting tubules and the related vasa efferentia, however, contain almost exclusively the mature spermatozoa indicating a resistance to liberation of the earlier maturation stages. This resistance was not in evidence with the gonads of younger stages. Sperm release is independent of any overt sex behavior on the part of the male, being a direct response to the anterior pituitary hormone stimulation.

Discussion

The presence of smooth muscle fibers in the ovaries of the frog has been demonstrated (Rugh, 1935) and the contraction of the lobes of the ovary has been seen in highly magnified moving pictures. It has further been demonstrated that the anterior pituitary hormone acts not directly upon the gonad but by way of either or both the nervous and the circulatory systems (Rugh, 1935 and Samartino and Rugh, 1945). This hormone, further, has been shown to stimulate other secondary sexual characters such as the thumb pad of the adult (Glass and Rugh, 1944) and the Mullerian ducts of the immature *Rana pipiens* (Schreiber and Rugh, 1945). In view of these and other findings, it is now clear that upon injection of the anterior pituitary hormone of the frog into the body cavity of the frog, supplementing its own glandular secretions, both the primary and secondary sexual characters are stimulated and the ovary (at least) utilizes its smooth muscle elements to free its follicles of their enclosed eggs.

Puckett (1939) and previously Swingle (1921 and 1926) found a race of bullfrog tadpoles in which there were so-called undifferentiated gonads, particularly in the male, known as protestes. These protestes reacted to daily injections of various mammalian hormones (pituitary and gonadal) by showing precocious development and differentiation, particularly when male sex and pituitary hormones were injected simultaneously. There was no influence on the differentiation of the gonads with regards to sex, but rather the pituitary treatment determined the rate at which the gonads grew. The variety of *Rana catesbiana* used in the experiments reported in this paper did not show the stage of undifferentiated gonads described by Puckett, but they did show a decided response to the amphibian pituitary alone. Adult female frogs have been found to be almost totally resistant to mammalian pituitary extracts (Rugh, 1935 and 1942), while the closely related toads and salamanders would respond readily.

Recently Schreiber and Rugh (1945) treated metamorphosing *Rana pipiens* with the adult frog anterior pituitary hormone and also with the female hormone estradiol benzoate. There hormones were used independently and it was demonstrated that the pituitary hormone acted on the gonad while the estradiol acted upon the gonaducts (oviducts and Mullerian ducts). It is therefore evident that the gonad, whether immature or mature, will respond to anterior pituitary hormone stimulation if the frog is treated with adult amphibian pituitary glands.

The period of this experiment, i.e., 48 hours, was too short to allow demonstra-

tion of any growth response of the gonads but there was immediate and drastic response at all stages, whether spermatozoa were present or not, by the elimination of cellular elements from the forming seminiferous tubules. Since there is experimental proof of smooth muscle presence and activity in the frog ovary, and since the post-stimulation picture of the frog testis indicates a greater response at the periphery, it is suggested that here too there may be pituitary stimulation of smooth muscle elements within and surrounding the testis which causes the evacuation of cellular elements. Even if there were evidence of selective action of the pituitary hormone on a specific cell type such an hypothesis would have no real precedent. We are dealing with phases in the maturation of one cell type and, in the immature gonad, all steps in maturation are found within the collecting tubules and the vasa efferentia.

Summary and Conclusions

1. Bullfrog tadpoles and immature male frogs of body lengths of 45, 65, 95, and mature bullfrogs of 120 mm. body lengths, possessing normally differentiating gonads, were treated with anterior pituitary glands from adult frogs of the same species.

2. Scattered spermatozoa normally appear shortly after metamorphosis but by the stage of 65 mm. body length there are relatively abundant structurally mature spermatozoa.

3. The short duration of the experiment, i.e., 72–96 hours, did not allow for any demonstrable growth response of the testis to the anterior pituitary hormone.

4. Even before mature spermatozoa appear, or there is any evidence of a lumen within the seminiferous tubule, the testis of the immature bullfrog reacts to the anuran anterior pituitary hormone by evacuation of cellular elements of the forming seminiferous tubule, and by the formation of exaggerated lumina.

5. Cellular elements, found after pituitary stimulation within the collecting tubules and the vasa efferentia, consist of all stages in maturation except the spermatogonia. Whole cysts of similar cells have been seen, indicating rather violent evacuation of the gonad.

6. The more mature the testis the more resistant are the earlier maturation stages to anterior pituitary hormone release from the gonad, so that in the collecting tubules of the newly metamorphosed bullfrog testis there may be found all stages from spermatocytes to spermatozoa but in the tubules of sexually mature (adult) males there are rarely any cells except spermatozoa.

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INDEX

AMOEBA carolinensis (Wilson, 1900), 8. Amoebae, fresh water, the contractile vacuole and the adjustment to changing concentration in, 158.

Arbacia punctulata, dilution medium and survival of the spermatozoa of, 177.

Attachment and growth of barnacles, effect of water currents upon, 51.

Attachment and growth of Bugula neritina, toxic effects of copper on, 122.

Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera), 265.

BARNACLES, effect of water currents upon the attachment and growth of, 51.

BODENSTEIN, DIETRICH. Investigation on the locus of action of DDT in flies (Drosophila), 148.

Brachystethus rubromaculatus Dallas, the elimination of chromosomes in the meiotic divisions of, 19.

Bugula neritina, toxic effects of copper on attachment and growth of, 122.

CARLSON, J. GORDON. Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis, 109.

Chironomus salivary gland chromosomes, micrurgical studies on, 71.

Chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, the elimination of, 19.

Ciliates of the family Ancistrocomidae Chatton and Lwoff, I, 1.

Ciliates of the family Ancistrocomidae Chatton and Lwoff, II, 200.

Contractile vacuole and the adjustment to changing concentration in fresh water amoebae, 158.

Correlation between the possession of a chitinous cuticle and sensitivity to DDT, 97.

COSTELLO, DONALD P. The swimming of Leptosynapta, 93.

CUTKOMP, LAURENCE K. *See* A. GLENN RICHARDS, 97.

D'ANGELO, ETHEL GLANCY. Micrurgical studies of Chironomus salivary gland chromosomes, 71.

DDT, correlation between the possession of a chitinous cuticle and sensitivity to, 97.

DDT, in flies (Drosophila), investigation on the locus of action of, 148.

Dilution medium and survival of the spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration, 177.

Discocephalini (Hemiptera), autosomal elimination and preferential segregation in the harlequin lobe of, 265.

EFFECT of water currents upon attachment and growth of barnacles, 51.

Elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, 19.

GRAPHIC method of describing the growth of animals, 141.

Grasshopper neuroblast during mitosis, protoplasmic viscosity changes in different regions of the, 109.

HALL, C. E., M. A. JAKUS, AND F. O. SCHMITT. An investigation of cross striations and myosin filaments in muscle, 32.

HAYASHI, TERU. Dilution medium and survival of spermatozoa of Arbacia punctulata. II. Effect of the medium on respiration, 177.

HENLEY, CATHERINE. The effects of lithium chloride on the fertilized eggs of Nereis limbata, 188.

Heteroagglutinins in body-fluids and seminal fluids of various invertebrates, 213.

HOPKINS, DWIGHT L. The contractile vacuole and the adjustment to changing concentration in fresh water amoebae, 158.

INTRACELLULAR alkaline phosphatases, distribution and properties of, 220.

KOZLOFF, EUGENE N. Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tagularum sp. nov. and Crebricoma gen. nov., 1.

KOZLOFF, EUGENE N. Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmo-

tricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov., 200.

KRUGELIS, EDITH JUDITH. Distribution and properties of intracellular alkaline phosphatases, 220.

LEPTOSYNAPTA, swimming of, 93.
Lithium chloride, effects on the fertilized eggs of Nereis limbata, 188.
Locus of action of DDT in flies (Drosophila), 148.
LOOSANOFF, VICTOR L. AND CHARLES A. NOMEJKO. Feeding of oysters in relation to tidal stages and to periods of light and darkness, 244.

MARINE Biological Laboratory Library, serial publications added to the, 88.
Meiotic divisions of Brachystethus rubromaculatus Dallas, elimination of chromosomes in the, 19.
Method of describing the growth of animals, 141.
MILLER, MILTON A. Toxic effects of copper on attachment and growth of Bugula neritina, 122.
Myosin filaments in muscle, 32.

NEREIS limbata, effects of lithium chloride on the fertilized eggs of, 188.
NOMEJKO, CHARLES A. *See* VICTOR L. LOOSANOFF, 244.

OYSTERS, feeding of, in relation to tidal stages and to periods of light and darkness, 244.

PHYSIOLOGY of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia, 234.
Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis, 109.

RANA catesbiana, effect of the adult anterior pituitary hormone on the tadpoles and the immature male frogs of, 291.
RICHARDS, A. GLENN AND LAURENCE K. CUTKOMP. Correlation between the possession of a chitinous cuticle and sensitivity to DDT, 97.
RUGH, ROBERTS. The effect of the adult anterior pituitary hormone on the tadpoles and the immature male frogs of the bullfrog, Rana catesbiana, 291.

SALIVARY chromosome manipulation, 71.
SCHMITT, F. O. *See* HALL, JAKUS, AND SCHMITT, 32.
SCHRADER, FRANZ. Autosomal elimination and preferential segregation in the harlequin lobe of certain Discocephalini (Hemiptera), 265.
SCHRADER, FRANZ. The elimination of chromosomes in the meiotic divisions of Brachystethus rubromaculatus Dallas, 19.
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SHORT, ROBERT B. Observations on the giant amoeba, Amoeba carolinensis (Wilson, 1900), 8.
SMITH F. G. WALTON. Effect of water currents upon the attachment and growth of barnacles, 51.
Striations and myosin in muscle, 32.
Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). I. Hypocomina tagularum sp. nov. and Crebricoma gen. nov., 1.
Studies on ciliates of the family Ancistrocomidae Chatton and Lwoff (order Holotricha, suborder Thigmotricha). II. Hypocomides mytili Chatton and Lwoff, Hypocomides botulae sp. nov., Hypocomides parva sp. nov., Hypocomides kelliae sp. nov., and Insignicoma venusta gen. nov., sp. nov., 200.

TYLER, ALBERT. Natural heteroagglutinins in the body-fluids and seminal fluids of various invertebrates, 213.

VISCOSITY changes in grasshopper mitosis, 109.

WALFORD, LIONEL A. A new graphic method of describing growth of animals, 141.
Water currents and growth of barnacles, 51.
WILLIAMS, CARROLL M. Physiology of insect diapause: The role of the brain in the production and termination of pupal dormancy in the giant silkworm, Platysamia cecropia, 234.
Woods Hole Oceanographic Institute Library, serial publications added since February, 1943, 88.